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# THIRTY-FIRST ANNUAL MEETING OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

AMES, IOWA, DECEMBER 30, 1929 TO JANUARY 1, 1930

*Headquarters, Memorial Union Building, Iowa State  
College*

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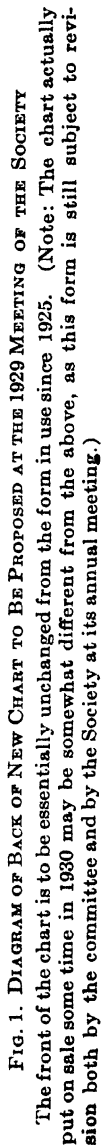
## ABSTRACTS

*Report of the Committee on Bacteriological Technic: New Undertakings During the Year 1929.* H. J. CONN, *Chairman*; VICTOR BURKE, BARNETT COHEN, ELIZABETH F. GENUNG, IVAN C. HALL, W. L. KULP, *Members*.

During 1929 this committee has begun a number of new undertakings which it seems should be called to the attention of bacteriologists in general.

Last winter a questionnaire was sent out to various users of the Society's Descriptive Chart to secure information as to ways in which the chart might be improved. Naturally, the questionnaire showed much difference in opinion, and it became obvious that it would be impossible to suit everyone. Nevertheless a certain number of changes were suggested with sufficient uniformity so that it was thought they could safely be incorporated into the next revision of the chart. These were as follows: (1) to include blanks for recording the pathogenicity and serologic properties of an organism; (2) to expand the table on fermentation to include other carbohydrates, alcohols, and glucosides, providing space at the same time for recording other observations besides those allowed on the last chart; (3) to provide blanks better adapted than at present for recording the action of bacteria on litmus and methylene blue in milk; (4) to eliminate to some extent the descriptive terms on the present chart, especially in the case of agar and gelatin colonies, and to allow the same information to be recorded by means of sketches. A revised chart embodying these features has been drawn up and is to be presented at the Society meeting in December 1929 (see fig. 1). Following the recent custom of the committee, the Society will not be asked to adopt the chart, but merely to allow the committee to have it printed and put on sale.

This Descriptive Chart, which was originally the chief concern of the committee, has now become only a small part of its activities. These activities, in fact, are becoming so various that it is impossible to obtain a personnel for the committee all of whom are interested in the various lines of work undertaken. For this reason it has been decided to assign each new committee member to a special field and to allow him at any time he desires to appoint a sub-committee in that field with himself as chairman. Thus at present Dr. Victor Burke represents the field of pathological technic, and Dr. Ivan C. Hall anaerobic technic. It is thanks to the former that the proposed revision of





the chart provides for recording the pathological and serological characteristics of an organism.

Dr. Hall, as committeeman on anaerobic technic, has drawn up a summary of methods for the isolation and study of anaerobic cultures which is shortly to be published as Supplement D to the Manual of Methods for Pure Culture Study of Bacteria. This supplement may even be issued before the present report appears in print. When ready, it will be announced in the advertising pages of this journal. It is to become a part of the Manual as sold in the future, and will be sent to all subscribers to the Continuation Service of the Manual. It will also be placed on sale by the committee in separate form for those who desire it without the rest of the Manual.

Attention is also called to the fact that the year 1930 is likely to see a fairly complete revision of this Manual. No announcement is ready as yet concerning the actual nature of the changes to be adopted, but those who subscribe to the Continuation Service of the Manual will receive the revised sections as issued.

In coöperation with other societies through the Commission on Standardization of Biological Stains, the chairman of this committee has this year revised the book *Biological Stains* published by the Commission. This book has been considerably enlarged. It includes a discussion of a considerable number of dyes not referred to in the earlier edition, and also gives the technic for about 75 of the more commonly used bacteriological and histological staining procedures. Announcement of this new edition of the book appeared in the advertising pages of the September number of this journal.

All the publications here referred to as sold by the Society or by the Commission on Biological Stains can be obtained from the chairman of this committee (Lock Box 299, Geneva, N. Y.).

## GENERAL BACTERIOLOGY

1. *Designation of the Relation of Bacteria to Free Oxygen.* D. H. BERGEY, University of Pennsylvania, Philadelphia, Pa.

The origin of the terms used in the literature is presented with the conflicting definitions for the terms in common use, as aerobic; anaerobic; aerobic, facultative anaerobic; anaerobic, facultative aerobic; aerophilic and microaerophilic.

Simplification of the terms used in grouping the facultative types is suggested in order to avoid confusion in the minds of students.

Bacteria classed by some authors as facultative anaerobic should be

termed aerobic, facultative; and bacteria classed as facultative aerobic should be termed anaerobic, facultative or microaerophilic.

2. *A Conductivity Method for the Analysis of Mixtures of the Volatile Acids.* E. E. MOORE, E. I. FULMER, AND R. L. FOSTER, Laboratory of Biophysical Chemistry, Iowa State College, Ames, Iowa.

It was shown that the antilogarithm of the specific conductivity of a mixture of two volatile acids is a linear function of the percentage composition. This relation was found for the mixtures formic-acetic, acetic-propionic, and formic-propionic acids in concentrations from 0.00641 N to 0.0127 N. The relation is

$$10^{S_{AB}} = m (\%B) + S_A \quad (1)$$

in which  $S_{AB}$  = the specific conductivity of the mixture, and  $S_A$  = the specific conductivity of the acid A at the same normality as the mixture. The per cent of acid A in a mixture with acid B can be calculated from the following relation

$$\text{Per cent A} = \frac{\text{Antilog } S_{AB} - \text{Antilog } S_B}{\text{Antilog } S_A - \text{Antilog } S_B} \times 100 \quad (2)$$

in which  $S_{AB}$  and  $S_A$  have the same meaning as in (1) and  $S_B$  = the specific conductivity of acid B at the same concentration as the mixture.

These relations are being applied to the analysis of the volatile acids produced by fermentation and to more complicated mixtures.

3. *A New Vessel for the Efficient Aeration of Bacterial Cultures in Liquid Media.* C. A. MAGOON AND B. C. BRUNSTETTER, Office of Horticultural Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture, Washington, D. C.

A new device is described and illustrated which makes possible the complete aeration of bacterial cultures in liquid media at all stages of development of the organism. The apparatus consists of a central culture vessel surrounded by a spiral tube which is a continuation of the culture vessel at the base and with which it communicates near the top. An air intake tube is inserted into the spiral tube near its base which delivers bubbles of gas into the spiral when aeration is in progress. As the bubbles of air or other gas ascend in the spiral the liquid is moved upward and is kept in continuous circulation. The gas escapes from the mouth of the culture vessel and may be collected readily for chemical examination. The design of the central vessel also facilitates the

removal of samples from the culture. Absence of obstructions and blind pockets assures thorough aeration of all portions of the culture at all times. This apparatus may also be used as a gas scrubber or gas absorption vessel in purely chemical investigations.

4. *An Aeration Train for the Study of Gases Produced in Bacterial Growth.*

HAROLD H. WALKER, Department of Public Health, Yale School of Medicine, New Haven, Conn.

In connection with experiments in bacterial metabolism, it was necessary to develop an apparatus for the determination of quantitative values of both  $\text{CO}_2$  and  $\text{NH}_3$  produced by the same culture. The total yield, free, dissolved and combined was desired. Since one of the products was acidic and the other basic, special arrangements were involved.

For the determination of free  $\text{CO}_2$  and  $\text{NH}_3$  a train for continuous removal by aeration during incubation was evolved. This consisted of a purifying train to remove atmospheric  $\text{CO}_2$  and  $\text{NH}_3$ , a Drechsel low-form gas-washing bottle for aeration of the culture during incubation at  $37^\circ$  in a water bath, and an absorption train. For absorption of  $\text{NH}_3$  a glass cylinder with Folin absorption bell was filled with distilled water sufficiently acidulated with  $\text{H}_2\text{SO}_4$  to absorb  $\text{NH}_3$  and prevent solution of  $\text{H}_2\text{CO}_3$ . Determination was made by nesslerizing an aliquot portion against standards containing a similar percentage of  $\text{H}_2\text{SO}_4$ . The second absorption unit in the train was a 250 ml. Pyrex Erlenmeyer flask with Brady-Meyer absorption tube, consisting of nine bulbs in series, each 32 mm. in diameter. This contained standard  $\text{Ba}(\text{OH})_2$  and following absorption of  $\text{CO}_2$  as  $\text{BaCO}_3$ , residual  $\text{Ba}(\text{OH})_2$  was titrated with standard  $\text{HCl}$  and phenolphthalein. Proof of complete absorption was afforded by a control consisting of a Bowen potash bulb filled with saturated  $\text{Ba}(\text{OH})_2$ , and located next in the train. Both purification and absorption portions of the train were further controlled by passage of the air through distilled water containing a pH indicator, which proved very useful. Safety bottles were included at each end of the line and were equipped with a specially evolved type of safety valve, which would permit discharge of an excess pressure without kick-back or cessation of the train. Either pressure or vacuum could be thus employed.

For the determination of dissolved or combined gases left in the medium after incubation and aeration, the following procedure was adopted. The culture was divided into large centrifuge tubes, the

shields of which were chilled in ice water to check metabolism. After centrifugalization, the supernatant liquid was divided into equal portions, one being treated with  $\text{H}_2\text{SO}_4$  to drive off all  $\text{CO}_2$  and the other with  $\text{Na}_2\text{CO}_3$  to drive off  $\text{NH}_3$ . For these operations two Van Slyke and Cullen aeration set-ups such as are commonly used for determination of urea-N, were employed. Standard alkali and acid respectively were used for absorption in the two set-ups and values were determined by titration.

5. *An Apparatus for Increasing the Amount of Inoculum without Diluting the Medium.* C. H. RAYBURN, C. H. WERKMAN AND R. M. HIXON, Departments of Chemistry and Bacteriology, Iowa State College, Ames, Iowa.

In many fermentations, the rate of growth of the organism is extremely slow. The rate can be increased by enlarging the amount of inoculum used, but in the general procedure this dilutes the medium to such an extent that the purpose of the increased inoculation is defeated. In the study of the propionic bacteria, this difficulty has been overcome by the design of an apparatus which permits the adsorption of the organisms on an inert agent and filtration from the medium. Fresh medium can be added to the sludge of organisms and adsorbing agent. Provision is also made for stirring the medium and for control of the gas.

In the study of the propionic acid fermentation, this apparatus has been run continuously for forty days. For the first media in the apparatus, ten days were required for the complete fermentation of the sugar. After the third renewal of the media, the fermentation would go to completion in 24 to 36 hours depending upon control conditions.

6. *The Growth of Bacteria in a Continuous Flow of Broth.* L. A. ROGERS, Bureau of Dairy Industry, U. S. Department of Agriculture, Washington, D. C.

A culture flask was arranged so that a continuous flow of fresh broth could be maintained at a rate which would change the medium about once every 24 hours. *Str. lactis* in a low sugar broth maintained a normal population for 15 days when the experiment was discontinued. *Es. coli* held a population above normal for 30 days. These two organisms together maintained high numbers for 18 days when the experiment was discontinued on account of contamination.

7. *A Comparative Study of the Effect of Cations on Bacterial Viability.*

C.-E. A. WINSLOW AND ELOISE T. HAYWOOD, Department of Public Health, Yale School of Medicine, New Haven, Conn.

The work here reported has confirmed previous studies from this laboratory in showing that various cations differ only quantitatively in their effect upon bacterial viability. All the cations studied stimulate growth in low concentration and inhibit it in higher concentration. Comparative tests in different media indicate that stimulating and inhibiting concentrations are about the same whether determined in distilled water, Dolloff's synthetic medium or pepton solution. Time studies show that the effect of a given cation concentration is nearly the same at all periods up to 144 hours although the inhibitive effect of toxic concentrations is somewhat less after 48 hours. Forty-eight hours was finally fixed upon as a standard test period using Dolloff's medium as menstruum.

For each cation there is a constant which represents its relative potency and this constant holds both in the stimulating and in the inhibitive zone. For the cations studied in detail the relative potency constants (expressed in molal terms) are as follows: K, 0.7; Na, 1.0; Mg, 1.5; Ba, 3.9; Li, 4.1; Ca, 9.0; Mn, 510.0; Zn, 814.0. The validity of these potency constants has been exhaustively tested by mixing various solutions and observing the effects produced. In general the theory of specific potencies has been fully confirmed without any necessity for involving "antagonism" phenomena.

8. *Preliminary Report on the Ergosterol Content of and the Effect of Ergosterol (Activated and Unactivated) on Growth of Mycobacterium tuberculosis.* PAUL S. PRICKETT, O. N. MASSENGALE AND WARREN M. COX, JR., Research Laboratories, Mead Johnson and Co., Evansville, Ind.

Because of the attention given to ergosterol by many investigators within the last few years search has been stimulated for sources of this substance. Since ergosterol is a sterol and closely allied to the lipid fraction of a material, the tubercle organism, which contains approximately 50 per cent lipid fraction, was investigated as a possible source of ergosterol. Two nonpathogenic cultures of *M. tuberculosis*, Nos. 599, bovine, and 607, human, from the American Type Culture Collection, grown on 5 per cent glycerol bouillon for periods of one week and one month, respectively, were found to contain no ergosterol. The same cultures when grown on 5 per cent glycerol-nutrient agar

slants to which activated ergosterol (potency 250,000 times that of cod liver oil) and unactivated ergosterol had been added in varying amounts from zero to 6.25 mgm. per slant showed equally good growths on all the slants.

9. *The Effect of Oxidizing and Reducing Agents on the Growth of Rhizobium spp. in Culture Media.* W. P. ALLYN AND I. L. BALDWIN, College of Agriculture, University of Wisconsin, Madison, Wis.

It has been shown that the growth of the root nodule bacteria of the Leguminosae and their reaction toward atmospheric oxygen depends to a certain extent upon the oxidation-reduction character of the medium.

In agar shake cultures of a rather highly oxidized medium, for example, mannitol-nitrate-mineral salts or ferric ammonium citrate media, these organisms establish a definite growth zone only under the surface of the medium. In a yeast extract-mannitol medium, which is somewhat more reducing, the organisms of cowpeas, soybeans and lupines grow under the surface; while those of clover and alfalfa show growth zones at the surface only. When 0.1 per cent cysteine is added to this medium, the growth line of all cultures is at the surface.

Definite stimulation is evidenced in petri plate cultures by reducing certain areas within a mannitol-nitrate or ferric ammonium citrate medium by means of agar blocks containing 0.1 per cent cysteine. Living potato blocks produce very similar results. Agar blocks containing iron reduced by hydrogen give slight stimulation.

Agar blocks containing ferric ammonium citrate definitely stimulate growth in a rather strongly reduced medium, such as yeast extract-glucose, produce less stimulation in yeast extract-mannitol, and a somewhat inhibitory effect in a mannitol-nitrate-ferric ammonium citrate medium.

10. *The Nature of the Fat-like Constituents of the Alfalfa Root Nodule Bacteria.* E. W. HOPKINS AND W. H. PETERSON, College of Agriculture, University of Wisconsin, Madison, Wis.

The root nodule bacteria of alfalfa, (*Rhizobium meliloti* No. 100), were grown on agar and in liquid media. After incubation for two to three weeks, the bacteria were washed from the agar slants, and the suspensions run through a supercentrifuge. The bacterial mass obtained was dried to constant weight and ground to a fine powder.

The ground cells were extracted, first with ether and then with chloroform: 0.6 to 1.2 per cent of ether extractable material was found, and 10.2 to 21.7 per cent of chloroform extractable substance.

The combined ether extract of four samples was examined qualitatively. It contained neither phosphatides (insolubility in acetone) nor sterols (Liebermann-Burchard test).

The chloroform extracted material was a brittle mass and had a high melting point ( $172^{\circ}$  to  $173^{\circ}\text{C}.$ ). Its saponification number was 603.8. The unsaponifiable matter contained only a trace of sterol. The greater part of the fatty acids liberated from the saponification mixture were soluble. These acids were separated into a volatile (66 per cent of total) and a non-volatile fraction (34 per cent of total). The volatile acid was identified as acetic acid by its Duclaux constants. Acetyl number and iodine number determinations indicated that the non-volatile fatty acids were saturated monohydroxy acids, and, as determined by titration, had an average molecular weight of 117.

*11. The Isolation and Some of the Characteristics of a Single Cell Culture of Nitrosomonas.* D. H. NELSON,<sup>1</sup> College of Agriculture, University of Wisconsin, Madison, Wis.

Owing to the fact that unsatisfactory results had been obtained when the plate method of Winogradsky was used for the isolation of the nitrite-formers, it was decided to attempt isolation by means of the single cell technic. For this purpose, enrichment cultures were prepared in the usual way by several successive transfers to fresh media. From these enrichments 365 cells were picked, of which 34 grew but did not oxidize the ammonium salt. It was clear that the enrichments must be improved, or the probability of securing a culture by this method would be rather remote. Two methods might be used: (1) eliminating every possible trace of organic matter from the cultures; or (2) finding some substance which would prove toxic to the contaminators, but which would allow the oxidizer to develop normally.

As a first step in this direction, the salts were recrystallized and the water redistilled from a dichromate-sulphuric acid solution in an all-glass still. This method alone proved insufficient to reduce the numbers of the non-oxidizers in the cultures materially. Next, crystal violet was tried, but it was found that the nitrite-former could not tolerate this dye, while the non-oxidizer was not materially affected. Copper carbonate was next tried. It was added with the  $\text{CaCO}_3$  after sterilization of the medium. This compound was found to reduce the numbers of the non-oxidizers materially, as shown by the reduction of the numbers of organisms developing on nutrient agar plates.

<sup>1</sup> National Research Fellow in Biological Sciences.

From one of these cultures about 20 single cells were picked and also a series set up having 2, 3, 4, 5, 6, 7, 8, and 10 cells in each culture. After 16 days, the 8-cell culture was found to be growing and oxidizing the ammonium salt; and after 21 days one of the single cell cultures was found to be growing and oxidizing. The single cell culture was reisolated by 56 single cell transfers, 2 of which grew and oxidized. This culture which had been isolated two times by the single cell technic was considered pure.

The organism is an ovoid rod about  $0.6\mu$  by  $0.9\mu$ , is motile, Gram-positive, stains readily with Czaplewski's carbol fuchsin, is often found in pairs, and does not grow in nutrient broth, nutrient agar or glucose-yeast water. In a shallow layer, for a three week period, it was found to oxidize 24.1 mgm. N per day per liter of medium.

12. *Spore Formation by Bacillus subtilis as Influenced by Pepton Concentration.* O. B. WILLIAMS, Department of Botany and Bacteriology, University of Texas, Austin, Texas.

Ratio counts at daily intervals of spore to vegetative cells in pepton water of various concentrations show that the percentage of spores in the more dilute media is greater than in the more concentrated. Direct microscopic counts show that the absolute number of spores in the stronger medium is greater than in the dilute.

13. *Special Methods in Marine Bacteriology and Preliminary Surveys of Pacific Waters.* HALDANE GEE, Scripps Institution of Oceanography of the University of California, La Jolla, Calif.

The author has introduced a bacteriological sampling device for use in deep water which can be operated in conjunction with other oceanographic instruments and collecting apparatus with a minimum loss of time. Storage of samples for examination on shore, which has been the chief criticism of studies on marine microorganisms, has been eliminated. This has been accomplished by the perfection of a solid medium technic which can be used on board ship in rough weather.

The preliminary bacteriological surveys indicate essentially marine conditions offshore at La Jolla. The total counts on Pacific water in the vicinity of Southern California indicate an even sparser population than has been reported for other ocean areas. Significant counts in the water are restricted to the topmost 25 meters, below which there is apparent sterility until the bottom zone is reached. Viable organisms in the bottom mud may be present to the extent of 20,000 per gram.



Some correlation has been observed between the viable surface count and variation in solar intensity throughout the day. The efficiency of the mediums used is at present undetermined.

Rods, molds and Actinomyces predominate in the flora isolated. Ammonia production is common and it is considered that bacterial activity may influence the pH relationships in the ocean and the calcium equilibrium.

14. *The Bacterial Proteolytic Enzymes: Their Activity at Different pH Values and the Effect of Sodium Chloride.* GEORGE SPITZER AND E. H. PARFITT, Purdue Agricultural Experiment Station, Lafayette, Indiana.

From our previous work on the study of bacterial proteolytic enzymes we deemed it desirable to carry on a systematic investigation, taking into consideration the influence of different degrees of acidity and also the influence on the activity of the enzymes of varying amounts of salt.

In this experiment we used *B. ichthyosmius* as a source of bacterial enzymes.

Two liters of peptonized milk were inoculated with the organism and incubated for seven days at room temperature. After incubation, sufficient toluol was added to saturate the solution and to leave a layer on top of the liquid. This was then held at room temperature to autolyze for ten days, after which the solution was filtered. Of this filtered solution 25 cc. were added to 200 cc. of sterile skimmilk and to 200 cc. of a 2.5 per cent solution of sterile gelatine, respectively. The media were then adjusted to the desired pH by the use of either sodium hydroxide or lactic acid. Then the contents of each flask were made up to 250 cc. with distilled water. After adjustment, 10 cc. of toluol were added to each flask. The media were then held at 37°C. At the end of 5-, 10- and 15-day periods chemical analyses were made to determine the nitrogen compounds not precipitated by phosphotungstic acid and by saturated solution of zinc sulphate. Prior to analysis each medium was checked for freedom from bacterial growth. The results are shown in the accompanying table.

From our data, it is evident that the pH value or acidity of the medium has a decided influence in controlling the hydrolysis of both proteins. The optimum lies between pH 6 and 8. There is a rapid fall of the curve as the pH decreases. At pH 4 there is a marked retardation, and at pH 3 little or no enzyme action occurs. The increase in nitrogen not precipitated by phosphotungstic acid and the increase in

peptons show unmistakable evidence of the presence of both ereptic and tryptic enzymes. Little if any peptic action is shown; this action ought to be evident in the acid range. The effect of salt on the retardation of enzyme action is not as sharply defined as might be expected. There was a decided weakening of enzyme action when the media contained 6 per cent salt, a greater effect at 10 per cent salt and a decided and sharp inactivation at 20 per cent salt concentration. The influence of salt on the inactivation was increased at the higher acidities.

*Digestion of milk proteins and gelatine by the enzymes of B. ichthyosmius*

Experiments A and B showing the per cent amounts of soluble nitrogen not precipitated by phosphotungstic acid and of peptons, both based on the total nitrogen in the media. Digestion period: 15 days.

NaCl	pH 8 0		pH 6 0		pH 4 0		pH 3 0	
	N not pre- cipitated by P.T.A.	Peptons	N not pre- cipitated by P.T.A.	Peptons	N not pre- cipitated by P.T.A.	Peptons	N not pre- cipitated by P.T.A.	Peptons

A. Milk proteins

per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
0	16 65	14 65	12 95	18.02	2 65	0 78		
2	20 61	13 33	10 28	9.69	0 45	1.60	0 48	0.00
4	14.40	18 19	10 43	16.22				
6	10 00	10 76	6 04	6.57	0 00	0 78	0 00	0 00
10	9.20	6.98	8 66	7.21	0 58	3.37		
20	7.82	5 19	3 35	2.05	0 00	0 19		

B. Gelatine

0	17.63	44 59	15 36	50 90	2 03	5 13	2 29	8 64
2	16 22	48 42	12 02	49 84	1.53	3 62	1 55	3 32
4	16 37	50 08	14 07	45.70	1.33	4 34	1 09	4 08
6	11.52	40 71	6 06	20 95	0 25	3 69	0 89	2 15
10	12.07	34 69	10 67	12 80	1 39	2 46		
20	11.88	37 22	1 88	5.34	1.20	0 00		

15. *Dissociation of Bacterium dysenteriae, Sonne type, as Influenced by Variations in Culture Medium.* STEWART A. KOSER AND NORMA C. STYRON, University of Chicago, Chicago, Ill.

Three different strains of the Sonne type of dysentery bacillus were used in all experiments. By varying the composition of the medium in which the organisms were grown it was possible to accelerate production of intermediate and rough forms.

Cultivation at 37°C. in 5 and 10 per cent solutions of Bacto pepton in distilled water usually produced a decided S to R change. Twenty per cent pepton was less effective, while in one per cent pepton the type remained remarkably constant and there appeared to be little tendency to depart from the ordinary S form, even upon prolonged incubation. Nutrient broth, with 0.3 per cent meat extract in addition to one per cent pepton, was more effective than the 1 per cent pepton in enforcing colony change. Meat extract alone in concentrations of 0.3 to 3.0 per cent did not greatly favor the S to R change.

A comparison of 7 different brands of commercial peptons, each in 5 per cent concentration, showed decided differences in the tendency to force dissociative changes. An initial acid reaction of pH 5.5 to 6.0, in pepton solutions or broth, seemed to retard the appearance of intermediate and rough forms until development of the culture had changed the pH to 7.0 or more. Growth in 5 per cent bile did not cause production of rough forms. Addition of bile to nutrient broth or to pepton seemed to add little to the effect of these solutions.

Cultures obtained from isolated R colonies appeared to be moderately stable when held on agar slants at either room or ice box temperature. Daily subculture of R forms on agar slants at 37°C. resulted in the production of small numbers of S forms after several weeks of successive transfers. Daily or twice daily transfers in glucose broth were quite effective in producing a reversion of R to S, and in some instances S colonies appeared as early as the 5th transfer. The process usually continued so that between the 10th and 25th transfer a complete reversion of R to S was obtained in most instances.

#### 16. *Dissociation of Acidoproteolytic Bacteria by Thermobiotic Culture.*

PROF. DR. COSTATINO GORINI, Città degli Studi, Milan, Italy.

In 1894<sup>1</sup> I described a thermophilic bacillus isolated from sterilized milk, growing at 37° to 65°C. (*Bacillus lactis thermophilus*).

Now, in continuation of my studies on the dissociation of the acidoproteolytes,<sup>2</sup> I have succeeded by thermobiotic culture in milk at 50° to 70°C. in dissociating my *Bacillus acidificans-presamigenes-casei* into thermophilic, thermotolerant and mesophilic strains. I have observed that strictly thermophilic strains are dissociable from the S type of the

<sup>1</sup> Giornale R. Società Ital. Igiene, 16, 5, 1894 (and not 1895, as many authors erroneously report).

<sup>2</sup> Gorini, J. Bact., 1929, 17, 1.

bacillus, while at most thermotolerant strains are dissociable from the R type.<sup>3</sup>

Assuming that the unstable S type corresponds to the youthful form of the organism, it seems very probable that thermophilia (as well as motility)<sup>4</sup> are properties of those cells which are in a certain phase of germination, with a particular state of the protoplasm.

17. *The Growth of Thermophilic Cellulose Decomposing Organisms on Agar.* P. A. TETRAULT, University of Wisconsin and Purdue University.

Tetrault's modification of the Morse-Kopeloff anaerobic chamber was employed. Viljoen, Fred and Peterson's medium, to which was added agar and cellulose in the form of ground filter paper or filter paper rounds, was used for most experiments. Incubation was at 60° to 65°C.

With the streak method of inoculation the following technic was followed. A layer of clear agar was poured in the bottom of the chamber and allowed to solidify. A thin layer of agar containing finely ground paper was then added and also allowed to solidify. A round of sterile filter paper may be substituted for this layer. Streak inoculations were made with a straight wire. A third layer of agar was then poured over the inoculated surface. If a filter paper round was used, this third layer was clear agar. After solidification of the agar the chamber was inverted, sealed and incubated.

With the dilution method cultures were made in the ordinary way. The agar containing finely divided cellulose was added to the diluted inoculum in the chamber and mixed by rotating. The chamber was sealed as before and incubated.

In both cases, growth was evidenced by the appearance of digested areas in the cellulose. No well defined colony could be seen. Transfers made from such areas to liquid cultures or to other agar cultures always resulted in digestion of cellulose.

18. *Cellulose Decomposition by Anaerobic Bacteria.* PHILIP B. COWLES, Laboratory of General Bacteriology, Yale University, New Haven, Conn.

The isolation of cellulose-decomposing anaerobic bacteria similar to

<sup>3</sup> Rend. R. Acc. Lincei, 1928, 8, 598.

<sup>4</sup> Rend. R. Acc. Lincei, 1928, 7, 689.

those described and studied in pure culture by Omelianski (1902) has not been mentioned in the more recent literature. In the present investigation several strains of organisms morphologically like Omelianski's have been obtained in pure culture from the feces of horses and man, and from soil. All are thin rods about  $0.4\mu$  in diameter and from 1 to  $7\mu$  long; they are sometimes slightly curved and often occur in pairs. Spherical terminal spores from 1.0 to  $1.5\mu$  in size are produced.

The preliminary enrichment medium used was the fecal extract cellulose broth described by Khouvine (1923). In this and in most media, however, pure cultures would not grow without the associative influence of some other organism. *Bact. aerogenes* was found useful for this purpose. Growth on solid media was finally secured by inoculating plates of infusion agar containing 1 per cent cysteine hydrochloride, pH 7.0, with bits of decomposing cellulose from an enriched culture. After several days incubation under strict anaerobic conditions discrete, transparent colonies about 0.5 mm. in diameter were formed. Inoculations from these colonies into beef infusion broth tubes containing a strip of filter paper caused fermentation of the cellulose. No development took place in infusion broth alone. Of 23 carbohydrates used with the broth, only dextrin, arabinose, xylose and cellulose were found to induce growth. Glucose was not utilized. The fermentation of cellulose by these bacteria is always accompanied by acid and gas formation. The gaseous products are hydrogen and carbon dioxide in the approximate ratio of 3:1.

19. *Evidence of Xylanase Production by an Actinomyces.* ROGER PATRICK, C. H. WERKMAN AND R. M. HIXON, Iowa Agricultural Experiment Station and Department of Chemistry, Iowa State College, Ames, Iowa.

A species of actinomyces isolated from soil was found to attack xylan prepared from corn stalks with the production of a carbohydrate, probably xylose, which was pentose-positive to Bial's reagent and to the phloroglucinol test. The material reduced Fehling's solution and formed an osazone, melting in the range of pentose osazones.

The actinomyces did not attack xylose or any of the common carbohydrates or alcohols. It is a non-chromogenic species with an optimum at 37°C. Grayish white, powdery, elevated, round colonies with a typical odor are formed on xylan agar media. Milk is digested without coagulation.

20. *The Relationship between the Carbon and Nitrogen Metabolism of the Butyl Alcohol Forming Organism.* P. W. WILSON AND E. B. FRED, College of Agriculture, University of Wisconsin, Madison, Wis.

Although the butyl alcohol forming organism cannot use ammonium salts as the sole source of nitrogen, it appears to prefer this inorganic form in the presence of protein nitrogen. Ammonium salts are utilized less rapidly in the presence of simple forms of organic nitrogen, such as pepton and amino acids, than in the presence of native proteins such as those found in corn. A limit of about 75 to 100 mgm. per liter  $\text{NH}_3\text{-N}$  as the chloride, sulfate or phosphate can be used without causing a decrease in the total yield of the solvents (acetone, butyl alcohol, ethyl alcohol) produced from the carbohydrate by the organism. In this case, the yield of ethyl alcohol is increased at the expense of the acetone. If, however, more than 100 mgm. per liter  $\text{NH}_3\text{-N}$  is supplied to the organism, the total yield of solvents is decreased and the yield of acetone rises. With ammonium carbonate, 175 mgm. per liter  $\text{NH}_3\text{-N}$  can be used without lowering the yield of solvents; the distribution of the solvents is not changed by this salt. These effects of the ammonium salts on the yield and distribution of the solvents are due to the acid set free when the  $\text{NH}_3\text{-N}$  is used. The action of the liberated acid is due in part to a lowering of the pH of the medium, but also to a specific action of the various acids.

21. *Fermentation of Pentoses by Certain Propionic Acid Bacteria.* MARION FOOTE, W. H. PETERSON AND E. B. FRED, College of Agriculture, University of Wisconsin, Madison, Wis.

Twenty cultures of propionic acid bacteria were tested with respect to their ability to ferment arabinose and xylose. Of these, thirteen were found to ferment arabinose and one to ferment xylose. The rate of fermentation varied with individual cultures. Two cultures, one fermenting both arabinose and xylose, the other only arabinose, were especially vigorous in their attack on the pentoses. These were selected for a quantitative study of the products formed, and a balance was made against the sugar destroyed.

The culture which fermented both arabinose and xylose did not give complete recovery in the fermentation of arabinose. This culture converted 30 per cent of the arabinose and 40 per cent of the xylose into volatile acids, 25 per cent of the arabinose and 50 per cent of the xylose into non-volatile acids, and 10 to 15 per cent of the sugar into carbon dioxide.

The culture which fermented only arabinose gave the following yields of products, based on sugar destroyed: volatile acid 70 per cent, non-volatile acid 20 per cent, and carbon dioxide 10 per cent. Whereas the ratio of propionic to acetic acid in the fermentation of glucose is about 2:1, in the fermentation of arabinose the ratio is about 1:9.

*22. Physiology and Classification of the Propionic Acid Bacteria.* C. H. WERKMAN AND SARA E. KENDALL, Department of Bacteriology, Iowa State College, Ames, Iowa.

The propionic acid bacteria show an interesting cultural and morphological behavior which serves to set them apart as a generic group.

*Propionibacterium* may be characterized as immotile, gram positive, catalase positive, non-sporulating facultative short rods. Gelatine is not liquefied, indol is not produced. Involution forms appear in acid cultures and under aerobic conditions of growth. Glycerol, erythritol, adonitol, glucose, mannose, levulose, galactose, and esculin are attacked by each of the 38 strains used. Dulcitol, perseitol, pectin, xylan and inulin are not attacked by any of the propionic bacteria. Propionic and acetic acids and carbon dioxide are produced from carbohydrates, glucosides, alcohols and organic acids.

As a source of nitrogen nucleic acid, yeast, or pepton may serve but ammonium salts are not utilized. Vegetable extracts stimulate growth. A key has been prepared for the ten species recognized.

*23. Bacterial Production of the Isomers of Lactic Acid and Their Identification.* R. J. ALLGEIER AND W. H. PETERSON, College of Agriculture, University of Wisconsin, Madison, Wis.

In order to obtain the isomers in a high degree of purity, quantities large enough for repeated crystallizations of their zinc salts are necessary.

Eight liters of medium containing 3 per cent glucose, 3.5 per cent malt sprouts and an excess of  $\text{CaCO}_3$  were used for each culture. Culture R is a strain of *S. lactis* that produced almost exclusively *d*-lactic acid; culture A is a strain of *L. leichmanni* that produced the *l*-form of the acid. After ten days incubation at 28°C. for culture R and at 37°C. for culture A, the fermentation was complete.

The zinc salts of the acids were prepared in the usual way by acidifying, extracting with ether, boiling with  $\text{ZnCO}_3$ , decolorizing, and crystallizing (at 37°C.). Several crops of crystals were taken, and these were purified by repeated crystallization. After two to four recrystallizations, the specific rotation reached a maximum. At a concentration of

4 per cent anhydrous zinc lactate, the following constants were obtained for the two salts:

$$[\alpha]_D^{20} = + 8.37 \text{ and } - 8.39$$

The authors believe that, because of the small quantities of salt available, most of the values reported in the literature are too low.

## SYMPOSIUM

### RECENT ADVANCES IN VIRUS DISEASES

1. *Studies on the Nature of Bacteriophage.* J. BRONFENBRENNER, Department of Bacteriology, School of Medicine, Washington University, St. Louis, Mo.

A systematic study of the properties of bacteriophage failed to substantiate its living nature. The bulk of evidence seems to indicate that bacteriophage is an inanimate chemical product of bacterial metabolism. This substance seems to possess no lytic properties of its own. It seems to exhibit stimulating effect on homologous or closely related bacterial species. As a result, the rate of intracellular metabolism is abnormally increased with a consequent increase in osmotic pressure within the cell, and if water is available in the surrounding medium, bacteria take it up, swell, and finally burst. The intracellular hydrolysis preceding and causing the swelling and bursting of bacteria is indicated by the marked increase in concentration of the products of hydrolysis of bacterial protein in the medium. However, if water in the surrounding medium is immobilized by the addition of hydrophilic colloids, the swelling and bursting of bacteria is prevented. Thus, instead of visible lysis under these conditions there is observed, on the contrary, a rapid multiplication of bacteria due to the addition of phage. The active principle (bacteriophage) is present in the medium in the form of discrete units. This is due to ready adsorption of the bacteriophage on colloidal aggregates of the medium and not to organized nature of the agent. The particles thus carrying the active agent vary in size to a great extent. This circumstance permits the demonstration of the detachment of the active principle from some particles and its transfer to others. When purified, bacteriophage-containing solutions fail to respond to most sensitive tests for protein, even when concentrated to 1/1000 of the original volume. If concentration is carried on to dryness, it can no longer be brought in solution and its lytic activity is lost. Chemical analysis of the residue thus secured indicates that it



may be a carbohydrate containing a small amount of nitrogen either in its molecule or as an impurity.

2. *The Problem of Seed Transmission of the Typical Mosaic of Tobacco.*

B. M. DUGGAR, University of Wisconsin, Madison, Wis.

Continuing experiments with various substances that might be concerned in the adsorption of the virus of typical tobacco mosaic, experiments were made employing a variety of proteins and other complex substances. While the results were varied, they were in certain instances positive and this has led to a study of the influence of ground seeds of various types in inactivating the virus. The experiments have included seeds of diverse composition, that is, those primarily starchy and those primarily proteinaceous, representing several different families of plants.

Inactivation of the virus has been marked with certain proteinaceous seeds, but inactivation is not a factor of the absolute protein content, depending apparently upon specific proteins or other specific materials accompanying them. The ground tobacco seed is likewise capable of producing inactivation, but this is never complete at the concentrations employed. The probability of relation of transmission to adsorption and inactivation through storage proteins is pointed out.

3. *The Antigenic Properties of the Ultramicroscopic Viruses.* E. W. SCHULTZ, Stanford University, California.

A summary of the results of recent studies on the antigenic properties of the ultramicroscopic viruses, with special reference to the fundamental nature of the antigen-antibody reactions of ultraviruses.

4. *The Concentration of the Virus of Epidemic Poliomyelitis.*<sup>1</sup> PAUL F.

CLARK AND JOHN A. SCHINDLER, University of Wisconsin, Madison, Wis.

The brain-cord filtrate from cases of experimental poliomyelitis has been concentrated from 20 to 100 times by distillation *in vacuo*, until there is more virus per unit volume in the residual "concentrate" than in the original 5 per cent brain-cord suspension. This method lends itself to a number of possible studies on the nature of the virus. Some of those which have been accomplished may be itemized as follows:

1. Similar concentration procedures applied to the feces of monkeys fed with brain-cord suspensions show that many infective doses of the viruses will pass through the intestinal tract of these animals unaltered.

<sup>1</sup> This work was made possible through aid received from the International Infantile Paralysis Committee from funds given by Jeremiah Millbank.

The possible importance of this finding from an epidemiological point of view, is apparent.

2. The saturated solution of sodium chloride which results from the concentration of filtrate 36 times or more, does not destroy the poliomyelitis virus for at least four or five months, whereas the streptococci which have occasionally been isolated from the poliomyelitis cases, are destroyed in one month under similar treatment.

3. Careful microscopic examination of direct smears of the concentrate, and cultures by a variety of procedures have never shown the presence of streptococci or anything which we could definitely call "globoid bodies."

4. The "concentrate" may be subsequently dialyzed against sterile distilled water without destroying the virus, thus making it possible to use large volumes of the dialysate for animal work.

5. The washed sediment or globulin fraction which is obtained as the result of dialysis does not contain virus sufficient to infect a monkey.

6. Further separation of the recognized proteins by chemical precipitation of the dialysate shows that the virus is present in the water-soluble protein fractions, chiefly in the pseudo-globulin portion.

5. *Studies on Aphid Transmission of Plant Viruses.* ISMÉ A. HOGGAN,  
Tobacco and Plant Nutrition, U. S. Department of Agriculture.

The determination of the rôle played by various aphids in the transmission of specific plant viruses is of both fundamental and practical interest. That the virus of ordinary tobacco mosaic is transmitted by aphids has recently been laid open to question. Greenhouse trials have now demonstrated that *Myzus pseudosolani* Theob. and *Macrosiphum solanifolii* Ashm. are capable of transmitting this virus from tomato to various solanaceous hosts, although these aphids are apparently incapable of transmitting the same virus from tobacco. On the other hand, they will readily transmit the cucumber mosaic virus from both tobacco and tomato, as will also another species, determined as *Myzus circumflexus* Buckt. This latter aphid also appears unable to transmit the virus of ordinary tobacco mosaic from tobacco, in this respect resembling the peach aphid (*Myzus persicae* Sulz.). No adequate explanation can yet be offered to account for this peculiar selective capacity of the aphids both with respect to the virus and to the host plant.

It therefore appears that, while *M. pseudosolani* and *M. solanifolii* may be factors in the dissemination of ordinary tobacco mosaic on

tomato, none of the aphids studied are likely to be of importance in the dissemination of this disease in tobacco fields.

6. *Cytological and Bacteriological Investigations of Bean Mosaic.* RAY NELSON, Michigan State Agricultural Experiment Station, East Lansing, Michigan.

A cytological study has been made of two kinds of mosaic, mottle type common on pea bean and rugose mosaic on Refugee. Freehand, unstained sections from petioles mostly have been used, supplemented by paraffin sections stained with iron haematoxylin and Giemsa stains. The presence of a minute organism has repeatedly been demonstrated in chloroplasts from diseased plants. It is often found in great numbers in the cytoplasm of phloem, xylem and parenchyma tissues. Invaded chloroplasts show first a small vacuolized area containing the organism. The chloroplasts are eventually destroyed and the organism is found in abundance in the plastid detritus. The organism may be localized or widely distributed in the plant. It is apparently a minute coccus occurring usually in single, diplo or chain form but exhibiting considerable pleomorphism.

Using modified bacteriological methods successful isolations have been made from very young seeds taken aseptically from immature pods and from leaf tissues from mosaic plants grown under sterile conditions. The percentage of successful isolations from seeds apparently corresponds with the number of mosaic plants obtained from seed harvested from diseased plants. The organisms are sensitive to cultural conditions and frequent subculturing is necessary. Successful isolations and subcultures often fail without the aid of tissue cultures. Pathogenicity has not been proved and an organism morphologically similar has been isolated from highly susceptible varieties of beans showing no apparent symptoms of mosaic.

7. *The Toxicity and Germicidal Activity of Bacteriophage and of Chemical Disinfectants as Compared by An In Vivo Test.* JOHN E. WALKER, Research Laboratories, E. R. Squibb and Sons, New Brunswick, N. J.

Various dilutions of bacteriophage and of several chemical disinfectants were mixed with a definite quantity of staphylococci and injected intracutaneously in rabbits. The resulting lesions gave information concerning the germicidal activity of the substances and their comparative toxicity for animal tissues. The chemicals tested were mercuric chloride, phenol, formalin, tincture of iodine, and chloramine.

*Staphylococcus bacteriophage* had a much wider range of dilutions (from full strength to 1:512) over which the resulting lesions were partially suppressed without necrosis of the tissue. The nearest chemical in this respect was mercuric chloride, which could be diluted to one sixteenth of the strength causing necrosis and still partially suppress the *staphylococcus* lesion.

The results afford theoretical support for the view that bacteriophage is a more suitable local dressing for *staphylococcus* infections than are chemicals.

8. *Spread of Mosaic Virus in Tomato Plants.* W. A. McCUBBIN AND FLOYD F. SMITH, Pennsylvania State Department of Agriculture.

A report presented in *Science* (46: (1716), 486; November 18, 1927) indicated that the virus of tomato mosaic spreads through the stems at about the rate of 1 to 2 cm. per hour. Repetitions of this work have been carried out since that time and the results indicate that at somewhat lower temperatures than those first presented the passage of the virus is greatly delayed. The procedure involved rooting several branches of a tomato plant in surrounding pots so that any member of the resulting colony might be removed and grown independently; inoculation of one colony member with mosaic virus; removal of individual members thereafter at stated intervals; and subsequent observation of these to note the appearance or absence of mosaic.

When a section of the woody cylinder was removed from the stem connecting colony members the virus was apparently able to cross this point readily; and when a ring of cortex was removed at this point mosaic developed in the uninoculated shoot in several cases. It is believed that adequate precautions were taken to avoid accidental infection in these plants.

9. *Root Inoculation with the Virus of Tobacco Mosaic.* MAURICE MULVANIA, Southern College, Lakeland, Fla.

A number of experiments were conducted to determine the possibility of infecting tobacco plants with mosaic virus variously applied to the roots. Three general methods of procedure were employed as follows: first, the uninjured roots of young plants were placed in contact with juice of plants affected with the disease; second, needle inoculations were made into the rootlets; and third, the infectious juice was applied directly to the freshly cut roots.

In all, more than 150 plants were subjected to one or another of the

above processes. None of the plants showed any symptoms of the disease. Corresponding leaf inoculations gave from 80 to 90 per cent infections. Subsequent leaf inoculations into plants which failed to become infected through the roots, gave the usual high rate of infection common to leaf inoculations.

These experiments were conducted under somewhat unfavorable conditions, but the uniformity of results is significant.

## IMMUNOLOGY AND COMPARATIVE PATHOLOGY

1. *A Preliminary Report of a Spiral Pleomorphic Organism.* JEAN BROADHURST AND DOROTHY PEASE, Teachers College, Columbia University, New York.

This report is based upon the study of a highly pleomorphic organism, obtained from a throat culture, which grows readily under aerobic conditions upon ordinary laboratory media. Some phases of its vegetative, and possibly sexual, life history will be shown by lantern slides.

2. *The Effect of Heat Produced by an Ultra-high Frequency Oscillator on Experimental Syphilis in Rabbits.* C. M. CARPENTER AND R. A. BOAK, Department of Pathology, Union University, Albany Medical College, Albany, N. Y.

Forty-five rabbits have been injected intratesticularly with tissue extracts containing the Nichols strain of *Treponema pallidum*. From three to five days following the injection twenty-five have been treated three times a week for seven weeks between two electrodes in which was oscillating an alternating potential of 2000 volts at a frequency of 10,000,000 cycles. The resistance of the tissues of the animals' bodies to the passage of the waves raised their temperatures from normal to 106° to 108°F., which required a period of 30 minutes.

The twenty remaining rabbits which were left as controls developed, in every case, large edematous testes which finally broke down, forming chancres. Later the testes became adherent to the scrotum and appeared very hard and reddish black in color. In some instances the lower half of the scrotum sloughed off and gumma-like lesions developed. The pathological picture was typical of experimental syphilis, as described by Brown and Pearce.

With one exception, the testes of the group of rabbits that was treated showed no gross evidence of disease. Dark field examinations of extracts made from the testes of the treated group have failed to show

spirochaetes which were demonstrated in the various lesions of the control rabbits.

*3. Inoculation Malaria: Sexual and Asexual Strains.* NICHOLAS KOPELOFF, New York State Psychiatric Institute and Hospital, Columbia-Presbyterian Medical Center, New York.

Since June, 1923, the malarial treatment of general paralysis has been employed in over 450 patients. In a single strain of malaria continuously in use in over 300 patients, we have thus far failed to find any sexual forms of the parasite.

In order to determine whether or not a strain of malaria known to produce sexual forms will become asexual after repeated human passages a new sexual strain was carried along in parallel. This strain was first inoculated on May 9, 1928. A total of 123 patients—88 females and 35 males—have been inoculated (August 8, 1929).

It has been noted that after 6 months the capacity to produce gametocytes has been markedly diminished in female patients and to a lesser extent in male patients. Thus, during the first 6 months, of slides showing parasites in inoculated women, 69 per cent had gametocytes. During the subsequent 9 months only 16 per cent had gametocytes: a reduction of over 50 per cent. Furthermore, during the first 6 months of inoculation there were no female patients with malarial parasites showing a total absence of gametocytes, while there were 13 such instances in the 43 patients subsequently inoculated.

In male patients showing parasites during the first 6 months 73 per cent had gametocytes as against 38 per cent in the following 9 months: a reduction of 35 per cent. None with parasites showed a total absence of gametocytes in the first 6 months, and only one showed this absence out of 21 cases in the following 9 months.

The conclusion is that the capacity to produce gametocytes tends to diminish markedly in female patients and to a lesser extent in male patients. In all probability the sexual strain will become asexual on continued passage, but this result will be achieved much sooner in female than in male patients.

*4. The Biological and Clinical Significance of Diphtheroids in the Blood Stream.* R. KOCH AND R. R. MELLON, Institute of Pathology, The Western Pennsylvania Hospital, Pittsburgh, Pennsylvania.

The observations are based on the bacteriological findings in the blood cultures of hospital patients suffering from streptococcal infection. In

the light of the genetic relation between streptococci and diphtheroids, first demonstrated *in vitro* by one of us (Mellon) and repeatedly confirmed (Ramsin, DeNegri, and others) their presence in the blood stream of streptococcal cases can no longer be viewed as adventitious. In fact, the evidence suggests that the mechanism of recovery in streptococcal infection often involves dissociation of virulent streptococci into non-virulent diphtheroids, a process frequently synchronous with recovery of the patient (as indicated by febrile defervescence, euphoria, etc.). Moreover, their presence in the blood follows the disappearance of the streptococci which usually are recovered when the infection is clinically active; but when the infection is on the wane the diphtheroids appear.

The list includes four cases from each of which the primary cultures, taken early in the disease, yielded a *Streptococcus viridans* or a *Streptococcus non-hemolyticus*, but in the recovery period a diphtheroid. From two cases a non-hemolytic streptococcus in pure culture was grown which in stock culture changed spontaneously into the diphtheroid form. In another case the same diphtheroid was isolated at intervals five times; it was recovered as well from the spleen, removed at operation. The reticulo-endothelial system of this organ had phagocytosed these organisms in large numbers, testifying further to our belief that their presence was not accidental. Moreover, the histo-pathology of the spleen was indistinguishable from that found with subacute bacterial (streptococcic) endocarditis; nevertheless, a streptococcus stabilized in its conventional form has never been isolated from this case despite numerous attempts. From two cases the diphtheroid was grown twice, and from six cases the only blood culture taken yielded strains of cocco-diphtheroid or straight diphtheroidal forms.

We do not contend that loss of virulence by streptococci is of necessity accompanied by their *in vivo* transformation into diphtheroids. But the difficulty that often attends their cultivation from blood cultures suggests that the dissociation may occur without demonstrable evidence by the blood culture method in common use. When attention was properly focussed on this problem, we were able to demonstrate these organisms in a greatly increased per cent of the cases. This involves not only better and more varied technic in the laboratory, but the taking of blood cultures at a period when they are rarely called for, viz., during the convalescent or preconvalescent period. Properly stained smears will demonstrate them, even though they may not be viable. However, these are not included in the present study. By way of control, diphtheroids are of rare occurrence in the blood stream

in other types of cases with the exception of Hodgkin's disease. Here the presumption becomes increasingly strong that they are dissociates from the tubercle bacillus, a fact well known to occur *in vitro*.<sup>1</sup> Dissociation experiments by numerous workers under controlled conditions dissent from the view that the group of organisms spoken of as diphtheroids are derived solely from the diphtheria bacillus or its congeners. Final interpretation must await more prolonged study and more material.

5. *Streptococci in the Lesions of Experimental Poliomyelitis in Monkeys.*

EDWARD C. ROSENOW, Division of Experimental Bacteriology,  
The Mayo Foundation, Rochester, Minn.

Streptococci or diplococci are demonstrable only in or adjacent to lesions during the acute stage of the disease. They have not been found in sections of the brain and cord of normal monkeys, of those that have recovered from poliomyelitis, nor of monkeys that have died from other causes, such as tuberculosis and ulcerative colitis. The streptococci have been successfully demonstrated during the acute stage of the disease induced by intracerebral injection of emulsions and filtrates of fresh and glycerolated virus directly from human cases as well as from monkeys after a few to many passages. This has been true in some instances when cultures of the material injected proved sterile.

6. *Streptococci in the Spinal Fluid of Experimental Poliomyelitis in Monkeys.* EDWARD C. ROSENOW, Division of Experimental Bacteriology, The Mayo Foundation, Rochester, Minn.

Streptococci or diplococci, have been demonstrated with great regularity in smears from the sediment of the spinal fluid before and after death during the acute stage of the disease in monkeys. They are not found after recovery, during convalescence, in normal controls, nor in monkeys that have died from other causes such as tuberculosis, or ulcerative colitis. The streptococci have been successfully demonstrated in the spinal fluid when poliomyelitis was induced with emulsions and filtrates of fresh and glycerolated virus directly from human cases as well as

<sup>1</sup> It is claimed that diphtheroids are found in the blood of agonal cases, but probably not more frequently than numerous other microbic invaders. Here they are, of course, not related to the disease *per se* any more than are staphylococci, *E. coli*, and other agonal saprophytes. In this connection it is certain that these species are not isolated from active or convalescent cases of streptococcal infection.



from monkeys after a few to many passages. This has been true in some instances even when cultures of the material injected proved sterile.

7. *Bacteriological Findings in 186 Human Autopsies with Special Reference to the Anaerobic Bacilli.* IVAN C. HALL, Department of Bacteriology and Public Health, University of Colorado School of Medicine, Denver, Colo.

A summary is presented of a study covering a period of more than four years, undertaken to determine the incidence of both aerobic and anaerobic bacteria in the heart blood, and, when indicated, in meningeal, pericardial, pleural, and peritoneal exudates, also in abscesses whenever found, and occasionally bile and urine. The findings are analyzed in relation to the *postmortem* diagnosis, and in relation to the number of hours elapsing from death to autopsy. An effort is made to interpret the organisms found with respect to the clinical history of the patient and to the possibility of *postmortem* invasion.

8. *Further Studies on Canine Anaphylaxis.* NOBLE P. SHERWOOD AND O. O. STOLAND, Departments of Bacteriology and Physiology, University of Kansas, Lawrence, Kan.

Our experimental results seem to warrant the following conclusions:

1. We have confirmed the observations of Pearce and Eisenbrey that occasionally a dog is found not sensitive during the period of 14 to 21 days after the second sensitizing injection of antigen. We followed the technique recommended by Manwaring and used horse serum as antigen.

2. We have observed that some dogs will become sensitive as early as the 9th day after the second sensitizing dose.

3. A few dogs were found to be sensitive as long as 9 months after the sensitizing injections.

4. We have observed noticeable variations in prolongation of clotting time. In one very sensitive dog the clotting time was found to be normal when tested 5 minutes after the shocking dose and during the period of low blood pressure. In several dogs it was only moderately prolonged while in many blood failed to clot after standing several hours or over night. This suggests different mechanisms as in pepton shock.

5. We failed to observe any correlation between precipitin content and sensitizing antibody. This is in harmony with Manwaring's observations.

6. In experiments on passive sensitization we have found that dogs of apparently the same weight and age when given the same amount of sensitizing antibody vary from complete refractoriness to extreme sensitiveness when tested 48 hours later.

9. *The Sensitization of Cattle to Tuberculin by Non-Tubercle, Acid-Fast Bacilli of Bovine Origin.* E. G. HASTINGS, B. A. BEACH, AND ISABEL THOMPSON, University of Wisconsin, Madison, Wis.

From the tissues of cattle which reacted to tuberculin, and in which no lesions of tuberculosis could be demonstrated on post mortem examination, acid-fast bacilli have been isolated which are proven not to be tubercle bacilli by the injection into guinea pigs, rabbits and fowls. Tuberculosis-free cattle injected with these cultures are sensitized for periods varying from a few weeks to at least nine months to tuberculin injected intradermally or subcutaneously.

10. *Skin Reactions Following Intradermal Injections of Vaccines Prepared from Intestinal Bacteria. A Preliminary Report.* L. W. FAMULENER AND JOHN S. DAVIS, St. Luke's Hospital, New York.

These studies were undertaken to evaluate the intradermal test in relation to vaccinotherapy. Vaccines were prepared from stools of a large series of cases admitted to the Medical Services of the Hospital, including a group of patients, chiefly adults, but also several babies and children, suffering from various diseases. Bacterial cultures were made from stools, and the aerobic types of organisms were isolated for the preparation of vaccines. As a basis for comparative readings all vaccines were prepared and standardized by one method. Vaccines were chiefly prepared from *B. coli* types, *B. enteritidis*, enterococcus types, and more rarely from atypical Gram-negative bacilli, proteus, and streptococcus types. In most cases tests were made upon the patients from whom the organism was isolated, usually only once, but in some instances the test was repeated some days later. Cross-tests were made between members of the group, and on some who furnished no vaccine. The intradermal tests were made by the usual procedure, usually on the volar surface of the forearm, although other skin areas also were tested. Readings of the reaction were made over variable time periods. Vaccines prepared from organisms belonging to the *B. coli* group gave most marked skin reactions. Only one individual in the series failed to respond positively to this organism.

Vaccines prepared from other bacterial species derived from the stool ordinarily gave only slight reactions. In general the site of the injection seemed to play no part in the intensity of the reaction. Reactions in children were more severe, but subsided very rapidly. Repeated tests on the same individual usually showed a slightly greater reaction in the second series than in the first test. Blondes appeared to react more markedly than brunettes. Those who reacted strongly to their own vaccines usually reacted strongly to similar vaccines from others. Patients suffering from cardiac disease appeared to react to the tests more severely than those suffering from other conditions, although those subject to "intestinal upsets" usually reacted more severely than others, in particular with the vaccines of the *B. coli* type of organism.

The chief value of the routine use of the "skin test" with vaccines prepared from the *B. coli* group of organisms would appear to be as a control of the amounts of the various killed organisms which should enter into an autogenous polyvalent vaccine, and as a guide for its therapeutic use in the patient.

*11. Antibody Production in Allergic Rabbits.* C. A. BEHRENS, Purdue University, Lafayette, Ind.

Our observations have led us to believe that practically all of the rabbits that die when inoculated intravenously do so following the initial series of injections, irrespective of the dosage used. That is, practically as many rabbits die following 0.1 cc. intravenous injection as die after 1 cc. or even 5 cc.,—ten and fifty times the dose, respectively.

We have also noted that antibodies with higher titres are produced after injections with larger doses of antigen than with smaller amounts, consequently if possible, it is more advantageous to use the former dose than the latter. We know that rabbits that survive the shock of the first series of injections, stand subsequent inoculations with an antigen much more easily and undergo less physical deterioration, which is so essential for antibody formation, especially, if they have been permitted to recover from the effects of the initial treatment.

Furthermore, antibody development is as good in such allergic rabbits as in animals receiving the same antigen for the first time (controls) and in some instances, depending upon the nature of the foreign protein used, much greater than in untreated normal ("clean") rabbits. Stimulation with a second antigen gives rise to antibodies

for the first immunogen though they are never as potent as those originally elaborated.

In other words, the general trend is for greater antibody production with less physical strain and without fatalities.

12. *Studies on Fungi of the Genera Candida, Cryptococcus and Mycoderma Isolated from the Skin of an Epidemic Perlèche.* ERNST PRIBRAM, Department of Bacteriology and Preventive Medicine, Loyola University, Chicago.

Perlèche is a microbic contagious disease attacking the labial commissures of the mouth of children, resulting in a desquamation of the epithelium. Yeast-like organisms were isolated in 77 of 100 cases of an epidemic in Chicago. The studies of the microorganisms revealed three different genera of the family Oosporae: *Candida*, *Berkhout*, *Cryptococcus*, *Kuetzing* and *Mycoderma*, *Desmazières*. Cultures and microphotographic lantern slides will be demonstrated. The morphology, classification and the fermentative qualities of the microorganisms will be discussed.

13. *Demonstration of a Mold Isolated from the Hair of Children Affected with Ringworm of the Scalp.* ERNST PRIBRAM, Department of Bacteriology and Preventive Medicine, Loyola University, Chicago.

From the hair stumps of children affected with a ringworm of the scalp a mold has been isolated. The mold has been found four times in an epidemic in Chicago in 1927 and has also been isolated in 1929 from the hair of a child from Kansas City. The mold has a pigmented mycelium which forms brown spindle-shaped septated spores growing in chains. Demonstration of microscopic slides. The mold may be classified with the family Dematiaceae, subfamily Phaeodictyeae, genus *Alternaria*. A differentiation has been outlined between the genus *Alternaria* and other related molds isolated in ringworm of the scalp or of the skin. The pathogenicity of molds of the family Dematiaceae will be discussed.

14. *Effect of Preservatives on Agglutinating Serum.* ARLYLE NOBLE, Research and Biological Laboratories, Parke, Davis and Company, Detroit, Michigan.

Experiments were conducted to determine under identical laboratory conditions the effect of certain preservatives on agglutinating serum.

The antiseptics employed were glycerol (50 per cent), chloroform (1 per cent), mercuric iodide (1:10,000), chinosol (0.4 per cent), phenol (0.5 per cent), dibromin (0.4 per cent), ortho-chloro-phenol (0.2 per cent), trikresol (0.2 per cent and 0.4 per cent) and chloretone (0.5 per cent). The serum used was from one lot of antipneumococcic horse serum. Observations were made over a period of four years and three months on the agglutinin titre, appearance and sterility of samples of this serum kept at 6° to 8°C. and at room temperature.

Of the ten agents tested, 50 per cent glycerol proved the most satisfactory. The titre of the serum stored at 6° to 8° remained unchanged over the period of observation and fell off slowly at room temperature. There appeared to be no change in the color of the serum and there was no precipitate. The samples tested did not become contaminated though they were opened many times.

There was little choice between 0.2 per cent trikresol and 0.5 per cent phenol. The agglutinin titres remained the same at 6° to 8°C. and decreased rapidly at room temperature, becoming negative in 15 months. Both serums were always slightly cloudy, and they reprecipitated after filtering. They remained sterile at both ice-box and room temperatures. A heavier precipitate was produced by 0.4 per cent trikresol and the agglutinins disappeared more rapidly.

Chloroform, mercuric iodide, dibromin and chloretone, in the amounts used, did not preserve the serum at room temperature.

Chinosol proved a good preservative and the agglutinin titres did not change. However, it caused a heavy precipitate which rapidly reappeared after filtration; and the serum became dark, brownish green in color.

Ortho-chloro-phenol (0.2 per cent) produced a muddy serum which lost titre at room temperature.

*15. Some Agglutination Studies with Meningococci.* JOHN F. NORTON  
AND NORMA H. BROOM, Department of Health, Detroit,  
Michigan.

These studies include tests on a number of commercial therapeutic antimeningococcic serums. The organisms used were isolated during the past year from cases of meningitis and from carriers. A large number of these organisms were tested with polyvalent diagnostic serum and with the four type serums obtained from several sources. Agglutinin absorption tests are recorded for a number of organisms. The results indicate a great variability of serums in their ability to agglutinate meningococci.

16. *Potato Extract Broth for the Typing of Diplococcus pneumoniae.*

NORA LARSON AND LUTHER THOMPSON, Section on Clinical Pathology, Mayo Clinic, Rochester, Minnesota.

The effect of the medium upon the agglutination of pneumococci has been studied. Antigens were grown in glucose-brain broth (0.2 per cent glucose), and in nutrient broth to which had been added just before use, unheated, sterile extract of potato to the amount of 1 cc. per 6 to 7 cc. of broth. Serum broth and blood broth were also used in certain cases. The cultures studied were 17 in number from the following sources: chest fluid 11; sputum 2; spinal fluid 2; blood culture 1; mastoid 1. All cultures which did not agglutinate under any circumstances are not included. All the media used were suitable for growing pneumococci, either from the original material or from colony transplants from blood agar, except that the more delicately growing cultures require a heavier inoculation on the potato extract broth.

The advantages claimed for potato extract broth are that it is cheap, readily available, easy to prepare, keeps well and is a clear medium without sediment. A clear medium is important in getting a growth free from extraneous material with which to carry out agglutination and bile solubility tests.

Of 17 cultures, 3 or 17.6 per cent were positive when grown in potato extract broth, but negative when grown in brain broth medium. Also the potato extract broth gave distinctly superior results in 4 other instances, making a total of 40 per cent in which the potato extract medium gave superior results.

17. *The Examination of Samples of Porcine Blood for Brucella abortus*

*Agglutinins.* R. A. BOAK AND C. M. CARPENTER, Department of Comparative Pathology, New York State Veterinary College, Cornell University, Ithaca, N. Y.

Agglutination tests for *Brucella abortus* infection have been made on approximately 4000 samples of pig's blood collected in abattoirs from various parts of New York State. A large percentage of the pigs had been shipped from the Middle West, principally from the states of Missouri, Iowa, Minnesota, Illinois, Indiana and Ohio.

A very low incidence of infection has been found as determined by the agglutination test. The samples of blood collected from New York State hogs have shown no more infection than those from the Middle West.

18. *Fermentation of Monosaccharids by Organisms of the Abortus-Melitensis Group.* MARION B. COLEMAN, HELEN H. OWEN, AND H. GLADYS DACEY, Division of Laboratories and Research, New York State Department of Health, Albany and New York City.

Previous observers have not recorded the fermentation of carbohydrates by organisms of the abortus-melitensis group although a few have reported that small amounts of glucose may be utilized by some strains. While studying the characteristics of this group at our laboratory, we found that acid production from certain monosaccharids might be demonstrated in a medium containing serum. Thirty-nine strains were tested, twenty-one of which were of bovine, twelve of human, four of porcine, one of caprine and one of unknown origin. Arabinose was fermented by all of the strains and xylose by all except one. The results with glucose, levulose and galactose varied. No reaction was obtained with any of the strains in medium containing rhamnose.

19. *Antigenic Studies of the Precipitation Test for Syphilis.* EMIL WEISS, Department of Bacteriology, Pathology and Preventive Medicine, Loyola University School of Medicine, Chicago.

An "optimal" antigen has been devised, based on the author's studies on lipoids. It is prepared by extracting dried and pulverized beef heart with acetone in the ratio 1:15 for one hour at icebox or room temperature. The extract is then filtered, the filtrate discarded, and the moist beef heart placed in the incubator at 37°C. for one hour (or longer) until dry. To the dried beef heart, 95 per cent alcohol is added, in the ratio 1:5. This extraction is carried out at 37°C. for 3 days. The extract is then filtered, and the filtrate is allowed to stand at room temperature overnight. This is done in order to facilitate the precipitation of lipoids, which are soluble in alcohol at 37°C., but not at room temperature. The extract is again filtered and 0.6 per cent cholesterol is added. This antigen is preferred to other secondary extracts, because the use of acetone in the first extractions avoids an excessive removal of suitable lipoids. The acetone soluble lipoids are useless for precipitation and represent only a fraction of the lipoids which are soluble in ether, chloroform, benzol and xylol.

The secondary extracts are prepared in the same manner as the "optimal" antigens, except that the first extraction is carried out with the above solvents and at ice box temperature for a briefer period. This is done in order to avoid an excessive loss of lipoids which are useful for the

precipitation. The secondary extracts are very satisfactory and rank next to the optimal antigen in usefulness. The plain alcoholic extract, prepared from beef heart also gives very satisfactory results. The ratio is 1:10 and the extraction is carried out at 37°C. for one hour. The extract is filtered, allowed to stand over night at room temperature, again filtered and 0.6 per cent cholesterol added.

The "optimal" antigen, the secondary extracts and the plain alcoholic extract have a wider specific zone than the antigens devised by Kahn. The preparation of these antigens is well defined. Variations in the results are comparatively small if mixtures of several beef hearts are always used. Complicated procedures as recommended by Kahn for the correction of his antigens appear superfluous.

*20. Precipitation Studies with Spinal Fluid.* R. L. KAHN, A. M. MALLOY AND LUCY WESTALL, University Hospital, University of Michigan, Ann Arbor, Michigan.

A series of studies was carried out with spinal fluid and its protein fractions employing the colloidal gold, mastic and Kahn precipitation reactions with the following results.

1. These three precipitation reactions depend on the globulin fraction of the spinal fluid. This fraction gives results which are equal to or slightly more sensitive than the original fluid.

2. The albumin fraction gives negative reactions with the colloidal gold and Kahn tests and practically negative reactions with the mastic test.

3. After performing a Kahn test with a solution of globulin obtained from spinal fluid and removing the precipitate by centrifugation, the residual globulin solution continues to give colloidal gold and mastic reactions.

4. Heating spinal fluid at 56°C. exerts little effect on the three reactions. The same applies to the heating of the globulin fraction. After heating the albumin fraction, it still gives negative reactions.

5. Higher temperatures up to 70°C. exert little effect on the reactions of the globulin solutions obtained from strongly positive spinal fluids. Globulin solutions obtained from moderately positive fluids turn from positive to weaker Kahn reactions after heating for 30 minutes at 65°C. and from positive colloidal gold and mastic reactions also to weaker ones after heating for 30 minutes at 70°C.



21. *On the Serological Diagnosis of Pullorum Disease in Domestic Fowls: The Chemical Nature and the Mechanism of the "Cloudy" Reaction.*  
GEORGE VALLEY AND E. P. CASMAN, Laboratory of General Bacteriology, Yale University, New Haven, Conn.

The phenomenon of the so-called "cloudy" or "false" reaction which is quite frequently met with in the agglutination test for pullorum disease was studied by analytical and simulative synthetic methods.

It was observed that in the agglutination tubes which showed the clouding or "false" flocculation the H-ion concentration was invariably increased in direct relation to the volume of opalescent serum added to the tube; i.e., the tubes containing 1 to 50 dilution of serum were always of lower pH than those containing 1 to 100 dilution. The magnitude of suppression varied. The "cloudy" reaction was prevented by adjusting the agglutination antigen to pH 8.5.

The flocculent material which was separated from the tubes exhibiting the "cloudy" reaction was positive to the biuret and xanthoproteic tests. Upon drying a yellowish brown, waxy mass was obtained which gave the usual fat test (oily spot) on paper. The presence of fat was further indicated by a positive acrolein test. The dry material was fractionated by ether-alcohol extraction. The ether-alcohol insoluble residue was found to contain from 14 to 15 per cent nitrogen. The ether-alcohol extract was further separated into two fractions: acetone-soluble (fat and cholesterol) and acetone-insoluble (lecithin and small amounts of galactolipins).

Synthetic studies in a system containing fowl serum, pullorum antigen and known quantities of fowl fat and lecithin showed that a complex resembling very closely the so-called "cloudy" precipitate could be obtained in antigen, serum and lecithin mixtures, either with or without fat. With serum, antigen and fat combination, without lecithin, the precipitate obtained was not typical. It appears then that the so-called "cloudy" reaction is due to the formation of a complex consisting of lipins and serum proteins, fat as such entering into the production of "cloud" as a result of adsorption.

The cloudiness and the increased acidity are apparently directly proportional to the amounts of opalescent serum used. It is, however, not evident that this acidity is the primary cause of the "cloudy" precipitation, although it is one of the contributing factors which render the conditions more nearly optimal for precipitation of protein-lipin complexes in the agglutination tube.

Evidence is presented to show that, aside from the metabolic changes accompanying egg laying, other factors, such as muscular exercise or

shock, may influence the pH and the lipid content of the serum and consequently bring about the so-called "cloudy" reaction.

*22. Studies on the Precipitin Reaction. II. The Composition of the Precipitate and Some Factors Influencing Its Formation.* CORA M. DOWNS AND SELMA GOTTLIEB, Department of Bacteriology and Immunology and the State Water and Sewage Laboratory, University of Kansas, Lawrence, Kan.

In 1926 Downs and Goodner showed that solutions of glucose and sucrose inhibited the formation of precipitate when antigen was added to its immune serum. A solution of precipitate in glucose or in weak alkali was shown to contain both antigen and antibody. As an extension of this work the following points have been determined.

1. The uterine horns of virgin guinea pigs have been passively sensitized by the injection of washed precipitate.

2. By the injection of washed precipitate, uterine horns are actively sensitized to the antigen used to produce the precipitate.

3. Uterine horns are also sensitized actively to rabbit protein present in the washed precipitate. This confirms the work of Weil and others on the presence of both antigen and antibody in the formed precipitate.

4. The precipitate has been found to be readily soluble in weak acid and alkali.

5. The following salts in molar solutions markedly inhibit the formation of a precipitate when antigen and antibody are mixed: LiCl, NaCl, KCl, NaCNS, KCNS,  $Mg(NO_3)_2$ ,  $Ca(NO_3)_2$ ,  $Sr(NO_3)_2$ ,  $Al(NO_3)_3$ ,  $Al_2(SO_4)_3$ ,  $Fe(NO_3)_3$ ,  $FeCl_3$ . The following salts show no inhibition: NaBr, KBr, KI,  $NaNO_3$ ,  $KNO_3$ ,  $Li_2SO_4$ ,  $MgCl_2$ ,  $CaCl_2$ ,  $BaCl_2$ ,  $MgSO_4$ ,  $Ba(NO_3)_2$ .

*23. Phenol Coefficient Tests by the Hygienic Laboratory and Department of Agriculture Methods.* GEORGE F. REDDISH, Lambert Pharmaceutical Company, St. Louis, Mo.

Four phenol-like compounds were tested for phenol coefficients by six different cooperating laboratories, using the Hygienic Laboratory and the Department of Agriculture Methods. As a result of this study it is found that the coefficients obtained by both methods are very close indeed,—well within experimental error. The average differences in phenol coefficient for each product by the two methods are as follows: 3, 0.5, 2, and 8 per cent. The average difference between the two methods obtained by all six laboratories on all products examined was 3.4 per cent.

This proves conclusively that the results obtained by the Hygienic Laboratory and Department of Agriculture Methods are practically the same on phenol-like compounds and well within the limits of experimental error.

24. *A Retention Test for Inhalants and Similar Preparations.* JEAN BROADHURST AND BEULAH E. LEHR, Teachers College, Columbia University, New York.

The wide range of substances now used as inhalants, nose and throat sprays, etc., suggested a comparison of their retention effects, as a probable measure of their persistence on membranes and their values in treating and preventing infections.

If to agar plates, the respective chemicals are added in small amounts (1 drop, 2 drops), these plates may be subsequently seeded with *Staphylococcus aureus* (cultivated according to Reddish's method for antiseptics) after any desired interval, and their retention values measured by the inhibition of bacterial growth. The retention values of such preparations, as shown by the accompanying lantern slides, range from no effect at all to complete inhibition, even when seeded with 1,000,000 or more staphylococcus organisms, as long as five days after contact with the preparations. The relative retention effect has no fixed relation to the immediate disinfectant value.

## AGRICULTURAL AND INDUSTRIAL BACTERIOLOGY

1. *A Method for Isolating Cultures of Rhizobium spp. Directly from the Soil.* O. N. ALLEN AND I. L. BALDWIN, University of Wisconsin, Madison, Wis.

Budinov, 1907, studied the chemotactic effects of certain cultural media on *Rhizobium* spp. and suggested that it might be possible to utilize this factor in isolating the organisms from the soil. Studies on Budinov's method with a new series of media have shown that it is readily possible to isolate the root nodule bacteria directly from the soil.

Several media were tested and of these yeast-water-mannitol exerted the most selective chemotactic effects on *Rhizobium meliloti* and *Rhizobium trifolii*, while yeast-water-xylose was the most effective on *Rhizobium japonicum*.

Capillary tubes (the upper end sealed) were suspended upright through floating corks on a mixture of 1 gram of the soil to be examined in 100 cc. of distilled water. One of these tubes was plated after 1 hour,

others after 12 and 24 hours on brom-thymol-blue yeast-water-mannitol agar. The plates poured from the capillary tubes after a period of 12 hours showed the largest percentage of colonies resembling cultures of *Rhizobium*. Such colonies were picked and streaked upon differential media, and the reactions studied. Those cultures which gave typical cultural characteristics were then used to inoculate sterile seed.

Out of 14 cultures judged to be *Rhizobium trifolii*, 12 formed nodules upon *Trifolium pratense*. In like manner, 15 out of 16 of the cultures judged to be *Rhizobium meliloti* formed nodules on *Medicago sativa*.

2. *A Study of Rhizobium japonicum Isolated from Various Soils.* WILLIAM H. WRIGHT (Late Associate Professor of Agricultural Bacteriology), W. B. SARLES AND E. G. HOLST, College of Agriculture, University of Wisconsin, Madison, Wis.

In previous work, Wright was able to classify the eight strains of *Rhizobium japonicum* which he studied into two main groups. Cultural characteristics and nitrogen fixing ability were used as criteria in making this grouping. By means of the agglutination test, these two groups were subdivided into four serological groups, two of which contained the majority of the strains tested.

In the present study, a total of 156 strains of *Rhizobium japonicum* was isolated from soils in which soybeans had grown. These soils were obtained from Japan, Manchuria, and from the states of Virginia, Mississippi, Florida and Louisiana. All of the strains isolated could be placed in two main groups according to their cultural characteristics. Six serological groups could be demonstrated by means of the agglutination test.

There was a marked tendency for strains from any one soil to fall into one serological and cultural group. Strains from other soils were classified in the same group, hence no classification based solely on geographical distribution could be made.

3. *The Spontaneous Culture and Silica Gel Plate Methods for Studying the Non-Symbiotic Nitrogen-Fixing Bacteria in Soils.* R. H. WALKER, J. W. SULLIVAN AND G. G. POHLMAN, Iowa State College, Ames, Iowa.

The methods developed by Winogradski for studying the nonsymbiotic nitrogen-fixing bacteria are described in detail and the results from their use are presented and discussed.

With soils from one series of plots, which have received different

fertilizer treatments and upon which corn has been grown continuously for the past fifteen years, very striking results were obtained. In the spontaneous cultures, the amount of growth varied from none upon soil from an untreated plot, to a very dense growth upon soil from a plot which had received applications of lime and manure. The amount of nitrogen fixed in seven days on the silica gel plates, when inoculated with 1 gram of soil from these two plots, was 0.54 and 11.05 mgm., respectively.

It seems that these methods will be of distinct value to soil bacteriologists in studies on the presence and activities of the aerobic non-symbiotic nitrogen-fixing bacteria. In addition, it is quite possible that they can be utilized as biological means for testing the fertility needs of soils. Their value in this respect is shown for soils deficient in lime and phosphorus.

4. *A Method for Determining the Number of Azotobacter Colonies in Soil.*

IRVIN H. CURIE AND HAROLD W. BATCHELOR, Ohio Agricultural Experiment Station, Wooster, Ohio.

The Winogradsky silicic acid plate method of determining the number of *Azotobacter* colonies in soil has been modified so as to make the procedure more rapid and less cumbersome without appreciably lessening its selectivity. The silicic acid gel is replaced by 1.5 per cent agar and the 20 cm. glass petri dishes by aluminum petri dishes. A stock salt mixture is prepared by grinding and mixing the inorganic nutrients recommended by Winogradsky. Two per cent of mannitol is used as a source of energy.

One hundred cubic centimeters of the nutrient agar are pipetted into each aluminum petri dish and allowed to solidify. One gram of soil particles which will pass through a 20-mesh sieve but be retained on a 40-mesh sieve is distributed evenly over the surface of the solidified agar. The plates are incubated at 28°C. for four days and the colonies counted. The medium is sufficiently selective to prevent appreciable growth of organisms other than *Azotobacter*.

This procedure has been applied to soils from the fertility plots at the Ohio Agricultural Experiment Station.

*Azotobacter* colonies have not been found to be present in soils having a pH value less than 6.0.

In general the *Azotobacter* population has been greater in the check plots than in the plots receiving fertilizer additions.

5. *The Chemical Action of Aerobacter faeni on Xylose and on Sucrose.* C. R. BREDEN, E. I. FULMER, C. H. WERKMAN AND R. M. HIXON, Departments of Chemistry and Bacteriology, Iowa State College, Ames, Iowa.

The work reported is part of a general project on the utilization of agricultural wastes, especially the cornstalk, by fermentological processes. The organism used was *Aerobacter faeni*, and it was chosen because of its good growth on synthetic media with ammonium sulphate as a source of nitrogen. The chemical action of this organism on xylose and sucrose is being studied quantitatively on various synthetic media under variable conditions. Work up to the present shows the production of the following compounds from both xylose and sucrose: formic, acetic, succinic and lactic acids; ethyl alcohol; CO<sub>2</sub>; H<sub>2</sub>; 2,3-butylene glycol and acetyl-methyl-carbinol.

6. *Some Fermentation Characteristics of Various Strains of Rhizobium meliloti and Rhizobium japonicum.* R. H. WALKER AND P. E. BROWN, Iowa State College, Ames, Iowa.

Twenty-three strains of *Rhizobium meliloti* and twelve strains of *Rhizobium japonicum* were studied to ascertain the constancy of their fermentative characteristics and also the extent of the fermentative variations between individual strains of the same species. These organisms were grown in yeast-water-glucose and yeast-water-galactose media in five or more consecutive tests. The change in hydrogen-ion concentration in the media was taken as a measure of the fermentative ability of the strain of organisms in that medium.

The results indicate that the fermentative powers of individual strains of bacteria are approximately the same in the various consecutive tests, and that the fermentative ability of a particular strain of organisms is a comparatively constant character. The data also indicate clearly that there are large variations in the fermentation characters of different strains of the same species of legume bacteria.

Some of the *meliloti* strains produced a distinctly alkaline reaction, and others produced a strongly acid reaction in media of the same composition. Some strains made the media a hundred times as acid as other strains of the same species. Some of the *japonicum* strains produced a very slightly alkaline reaction in the media while others produced a reaction almost ten times as alkaline. The majority of the *japonicum* strains produced a much more alkaline reaction than the majority of the *meliloti* strains, but this difference was not true for all individual strains.

The various strains of *Rhizobium meliloti* ferment glucose and galactose with about the same comparative results. On the other hand, the strains of *Rhizobium japonicum* do not appear to ferment these two sugars in the same manner.

These results indicate that the fermentation test with glucose and galactose under conditions similar to those followed in these experiments would not serve to give a distinct separation of organisms of these two species of legume bacteria.

7. *The Effect of Nitrate Fertilization upon the Relationship between the Host Plant and Various Strains of Rhizobium spp.* D. H. DUNHAM AND I. L. BALDWIN, Purdue University, Lafayette, Indiana and College of Agriculture, University of Wisconsin, Madison, Wis.

The physiological relationship between the host plant and certain strains of *Rhizobium* spp. was studied under varying conditions. Soybeans and sweet peas were used as test plants. Each of these plants was studied without inoculation and with two strains of *Rhizobium* spp. One of the strains in each case possessed the ability to induce a high fixation of nitrogen with the plant and the other induced little or no nitrogen fixation.

Plants were grown in a glacial sand containing very little nitrogen. In each group of inoculation treatments, three series of nitrogen treatments were carried: first, no nitrogen; second, a light application of nitrates, and third, a heavy application of nitrates but not enough to suppress nodule formation completely.

With both plants, the uninoculated series and those inoculated with the poor strain produced essentially the same growth, regardless of nitrate additions. No evidence was secured to indicate that the "poor" strains are definitely harmful to plant growth, but they should rather be classed as neutral. Nitrate fertilization reduced nodule numbers with both the "good" and "poor" strains, and also reduced the fixation of nitrogen with the "good" strains.

8. *Recurrent Periodism of Nitrogen Fixation and Denitrification in Incubated Soils.* HAROLD W. BATCHELOR AND IRVIN H. CURIE, Ohio Agricultural Experiment Station, Wooster, Ohio.

During three successive years soil samples from one hundred and twenty plots, comprising fertilized and non-fertilized, limed and unlimed plots, were each incubated at 28°C. for a period of six months. Analyses for total nitrogen including nitrate nitrogen were made every two weeks during the incubation period.

The results indicate that under the conditions of the experiments, nitrogen fixation and denitrification tend to follow a periodic curve whose wave length extends from twelve to fourteen weeks. Irregularities are present in the curve, however, and the futility of making a single analysis for total nitrogen in nitrogen fixation experiments is pointed out. A plea is made for the discarding of the single incubation period in soil biology investigations.

*9. Phosphate Assimilation by Certain Soil Molds.* P. E. BROWN AND F. B. SMITH, Iowa State College, Ames, Iowa.

Pure cultures of twenty soil molds were grown in solutions and in soil for 45 to 75 days, in the presence of phosphate. Eighteen of the organisms, after incubation, decreased the amount of water soluble phosphorus present, while two cultures showed slight increases, indicating a definite phosphate assimilating power in the case of the 18 organisms at least. No relation was apparent between the change in reaction and the assimilating power of the molds.

*10. The Assimilation of Nitrates by Soil Microorganisms.* F. B. SMITH AND P. E. BROWN, Iowa State College, Ames, Iowa.

The disappearance of nitrate nitrogen and its reappearance in organic form in soils treated with oat straw were noted.

Bacteria and molds isolated from such a soil were studied in pure culture.

The assimilation of nitrates in sterile soil inoculated with cultures of these organisms was measured.

*11. A Case of Local Microbiological Activity in Soil.* NATHAN R. SMITH AND HARRY HUMFELD, Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington, D. C.

It was reported at the meetings last year that green manure increased the bacteria in soils when composite samples were taken through the layer of decomposing green manure.

In the work reported here two plots of Leonardtown clay loam, one limed (pH 7.0) and one unlimed (pH 4.6) were treated with green vetch as green manure. This was placed in a layer 5 inches beneath the surface. Samples were taken every 2 inches with a soil tube, 0 to 2 inches, 2 to 4 inches and 4 to 6 inches. From the 4 to 6 inch sample, the green manure layer was separated. This included not more than  $\frac{1}{2}$  inch of soil on either side. Analyses were made for total plate counts, protozoa, ammonia, nitrates, pH and carbon dioxide evolution.



The total plate counts, protozoa and ammonia of the three layers of soil did not vary appreciably. On the other hand, in the green manure layer, the numbers of bacteria increased enormously, reaching in 4 days a maximum of approximately 11 billions per gram of green material in the acid soil, and 22 billions per gram in the limed soil. The green manure disappeared rapidly, and the counts decreased as the proportion of soil to green manure increased in the sample. The lack of an increase in numbers in the soil of the 4 to 6 inch layer surrounding the green manure layer was very unexpected. Numbers of protozoa increased in 2 and again in 4 days. They decreased with the numbers of bacteria and the disappearance of the green manure. Ammonia production correlated with the numbers of microorganisms reaching the maximum of 1060 p.p.m. on the fourth day.

Nitrification was active in the acid as well as in the limed soil and the nitrates accumulated in the surface 2 inches, causing a lowering of the pH there.

Carbon dioxide evolution from the soil under these conditions was directly proportional to the numbers of microorganisms and ammonia production in the green manure layer.

From a microbiological standpoint sampling a soil to a depth of six inches and composting gives only a partial picture of what is taking place in the soil. For experiments such as these, specific methods of sampling must be used to suit the conditions.

*12. The Bacterial Flora of Aseptically Drawn Milk.* W. DORNER, Swiss Dairy Experiment Station, Liebefeld-Berne, Switzerland.

The investigations here described were made at the New York Agricultural Experiment Station, Geneva, N. Y. Thanks are due to various members of the Staff of the Bacteriological Division for help and suggestions given.

The examination of the milk samples was made by the Burri technic, using as a measure a calibrated platinum loop holding 1 mg. The milk was smeared on the dried surface of an agar slant. The medium used for the slants was 2 per cent agar containing 1 per cent tryptophane broth, 0.25 per cent glucose, 1 per cent diluted yeast extract and 5 per cent tomato juice; pH 6.8. Incubation: 3 days at 30°C. Standard agar plates were also poured from all samples and incubated at 37°C.

The examination of 993 samples of aseptically drawn milk from 132 cows belonging to 6 herds located near Geneva gave the following results.

The calculated average bacterial count of the milk from each herd showed great variations. The lowest herd count obtained on Burri slants was 3730 per cubic centimeter, while the highest herd count was 9050 per cubic centimeter. The average count of the milk from all cows was 7080 per cubic centimeter. On standard agar plates, the average counts from the individual herds were lower, varying between 530 and 3850 per cubic centimeter, the general average being 2780.

Figures were obtained showing the distribution of the samples according to their count. It appears that a small number of samples containing relatively high numbers of bacteria are usually the source of the greater part of the bacteria in aseptically drawn milk.

Figures were obtained which indicate the proportions of various groups of bacteria in the total bacterial count. The most important fact discovered was that organisms which are identical with *Bacterium lipolyticum* Evans were found on Burri slants to be most frequent in the samples and most numerous in the mixed milk; 47.9 per cent of all the bacteria found in this milk belonged to this species while only 18.4 per cent were micrococci and 32.2 per cent streptococci. On standard agar plates the dominant group of organisms is clearly that of the micrococci. Rods identical with *Bact. lipolyticum* occur in small numbers on standard agar plates. Their share in the bacterial count is only 10.4 per cent against 47 per cent for the micrococci and 42.3 per cent for the streptococci. Only 8.8 per cent of the samples contained rods.

This failure of the most frequently occurring organisms in aseptically drawn milk to develop on standard agar plates suggests strongly that this medium and technic is not suitable for controlling the quality of certified milk. The Burri technic which is more rapid and convenient for control work is especially well adapted for use upon low-count milks. This suggestion is supported by the fact that there is no evidence these rods from fresh milk play any pathogenic rôle in animals or in man.

Figures were obtained which show the average bacterial count of samples containing bacteria of the various groups. It was found that the samples containing streptococci developed the highest average number of colonies (14,980 per cubic centimeter on Burri slants and 15,140 on standard agar). The samples containing rods identical with *Bact. lipolyticum* showed counts of 8900 on slants and 7750 on plates, while the micrococci were lowest with 5810 and 5610 per cubic centimeter.

13. *An Investigation of Thermophiles in Freshly Drawn Milk.* P. ARNE HANSEN, New York State Agricultural Experiment Station, Geneva, N. Y.

Milk was drawn aseptically from 118 cows, one sample from each quarter. The experiment was repeated until 836 samples were examined. One-tenth cubic centimeter of each sample was plated upon glucose agar with an addition of autolyzed yeast, and incubated at 56° and at 62°C. The total number of colonies upon 836 plates was only 143. Of these, 107 strains were examined for their thermophilic properties. Among the 107 strains only 14 facultative thermophilic spore-bearing rods and 2 *Actinomyces* were found. These were able to grow continually at a pasteurizing temperature of 62°C. Seven strains proved to be *Bacillus nondiastaticus* Bergey, and 6 strains were *B. thermoliquefaciens* Bergey.

There was no correlation between the type of organism and the particular quarter from which the sample was taken. Considering the number of cows, this failure to repeat findings is striking; and the most probable conclusion that can be drawn is that the thermophiles isolated were contaminations derived from the air in the barn. The smallest number of thermophilic organisms was found in the cleanest barn. This fact also agrees with the theory of contamination.

It is also interesting to note that of the four strains of *B. nondiastaticus* which Prickett isolated, three came from dairy feeds, and one from raw milk. Bergey isolated *B. nondiastaticus* from dust and soil. Prickett isolated *B. thermoliquefaciens* from raw milk, while Bergey isolated it from oats and manure. Such findings make it natural to expect these species of thermophiles in barn air, and they explain why these organisms were found in our samples as contaminations.

All of these observations indicate that the udder is not a source of thermophilic bacteria.

The complete report of this work will appear as Technical Bulletin No. 158 of the New York Agricultural Experiment Station.

14. *The Control of Thermophilic Bacteria in Pasteurizing Plants.* M. W. YALE, Department of Dairy Industry, Iowa State College, Ames, Iowa.

Studies were made of the development of thermophilic bacteria at five pasteurizing plants with pasteurizing runs of six to seven hours. Long flow holders, coil pasteurizers and the Electropure system of pasteurization were investigated.

The factors found favoring the development of thermophiles were (1) the presence of thermophiles in the raw supply; (2) repasteurization of returns from milk routes; (3) prolonged holding at pasteurization temperatures; (4) the cooking of milk solids on the walls of internal tube heaters and regenerative heater-coolers; (5) dead ends in pasteurizing equipment; (6) filter cloths, and (7) development of thermophiles within the pasteurizer itself. All but the last of these factors may occur, regardless of the type of pasteurizing equipment in use.

Due to variations in equipment and methods of plant operation, each plant had a different combination of factors affecting the development of thermophiles and presented an individual control problem. Faulty plant operation proved to be more important in the development of thermophiles than the type of pasteurizing equipment. Due to the high temperature employed, 162° to 164°F., Electropure pasteurization was the least favorable for the development of thermophiles.

Filter cloths were a factor only when milk was filtered at a temperature above 100°F. When used continuously from four to five hours for filtering pasteurized milk at a temperature of 143° to 145°F., enormous numbers of thermophiles developed on the cloths. This factor was brought partially under control by installing a fresh filter cloth after each two hours of operation.

With the coil vat pasteurizers, the effect of prolonged holding at pasteurizing temperatures was to decrease the 37°C. plate count although, due to the development of the thermophiles, the total number of bacteria actually increased, as was shown by the direct microscopic count.

Direct microscopic examination, incubation of agar plates at 62.8°C., and the methylene blue reductase test at 62.8°C. proved reliable for the detection of thermophiles.

Part of this work has been reported as Tech. Bul. No. 156 of the New York Agricultural Experiment Station, Geneva, New York, and the remainder will be made available in a future bulletin of that station.

15. *Studies on Thermophiles in Milk.* C. S. MUDGE AND M. L. THORWALDSON, College of Agriculture, University of California, Davis, California.

Pinpoint colonies are often present upon plates poured from pasteurized milk. This condition has been confused with the presence of thermophilic bacteria in milk; these being demonstrated by microscopic examination when milk is held at 60°C.

Such examination discloses an enormous and exceedingly rapid increase in bacteria. Such bacteria also show the presence of spores. Assuming this increase to be growth, and calculating the generation time for this growth, our own work—as well as that published in the literature—indicates that the generation time is from four to five minutes which, although admittedly possible, is highly improbable. It seems for this reason that the increase in bacteria demonstrated by the microscope is not growth; but such increases can be accounted for by assuming that the organisms, although undemonstrable by the usual microscopical and cultural methods, are present in the milk either as spores or as other forms (conidia?).

These abnormally large numbers of bacteria have been demonstrated in the 1:10,000,000 dilution in milk showing a plate count of 1000 and a direct count of 50,000. Leucocyte-like bodies which rise with the fat are found to be filled with deeply staining granules when the milk is subjected to the action of alkali which shifts the hydrogen-ion concentration to pH 12. The nature of these granules is still under investigation. It is suggested that two forms of spores exist; that the organism passes rapidly through a vegetative state from one spore to another; that the latter spore is dormant. This dormancy is difficult to break although the authors have succeeded in doing this by means of a combination of alkali, heat and rest. Such dormancy is a well-known fact with seeds. This dormancy further would account for the current ideas that the cultures become sterile on holding.

This is a preliminary report.

16. *Variations in Streptococcus lactis*. L. A. HARRIMAN AND B. W. HAMMER, Dairy Industry Department, Iowa State College, Ames, Iowa.

In previous work on *Streptococcus lactis* at the Iowa Agricultural Experiment Station, a general correlation was noted between rapid coagulation at 21.1°C. and proteolysis of milk. The results herein reported were secured in an attempt to determine whether or not the variations in the rates of coagulation, often evident among cultures of *S. lactis* picked from a plate poured with a pure culture, are correlated with differences in action on the milk protein.

*S. lactis* cultures from various sources were plated on whey agar and about 25 colonies from each picked into litmus milk; the milk was held at 21.1°C. and observations made on the rates of coagulation of the different tubes. One or two of the tubes coagulating early and, when-

ever possible, tubes in which coagulation was delayed were selected for study. The rates of coagulation of the selected strains were checked by inoculations in which fairly definite amounts of fresh cultures were used. In this way strains which differed greatly in their rates of coagulation at 21.1°C. were sometimes secured from the same original culture. One of the rapidly coagulating strains from each source was then studied as the original cultures had been and the procedure of selecting and plating rapid coagulators was followed through a series. Some of the platings yielded strains which were decidedly slower in their rates of coagulation of milk at 21.1°C. than the strains from which the plates were poured, while others did not.

The rapidly coagulating strains caused a pronounced increase in the amino nitrogen content of milk as shown by the Van Slyke method, while the strains which were very slow in their coagulation did not. The previously noted correlation between rapid coagulation at 21.1°C. and proteolysis of milk accordingly continued under the conditions studied.

From the results secured it appears that slowly coagulating strains of *S. lactis* can be split off from rapidly coagulating ones. The plating of the slow coagulators never yielded rapid coagulators, which suggests that the variation is not a reversible one.

17. *The Influence of Certain Milk Bacteria on Swiss Cheese.* L. A. BURKEY AND W. C. FRAZIER, Bureau of Dairy Industry, U. S. Dept. of Agriculture, Washington, D. C.

The kettle milk used in the manufacture of Swiss cheese was examined daily to determine the number and proportion of the organisms which might be important in their influence on the cheese. The groups of organisms considered as significant types for this study were *S. lactis*, sporeformers (aerobic and anaerobic), casein digesters, white and yellow udder cocci, aerogenes-like organisms, and other prominent types of bacteria which would easily be distinguishable by colony appearance.

The sporeformers, udder cocci, casein digesters, and anaerobes seemed to have little effect on the quality of the Swiss cheese, except when they were present in numbers large enough to indicate a gross contamination of the milk.

The presence of *S. lactis* in large numbers, especially in proportion to the total number of bacteria, usually resulted in a poorer quality of cheese. This effect might be increased by large numbers of other undesirable organisms or might be counteracted to a considerable extent by the presence in the milk of certain streptococci discussed below.

Three types of streptococci which may be present in raw milk were found to have a beneficial effect on the cheese, when they were used as a starter with the *L. casei* (39a) culture. These organisms are referred to as "C<sub>1</sub>", "C<sub>2</sub>," and "H." The "C<sub>2</sub>" and "H", which are probably strains of *Streptococcus thermophilus*, stimulate acid production and counteract gas formation in the press. "C<sub>1</sub>" has only a slight influence at this time. The three organisms are believed to have their source in manure.

The presence of the aerogenes type in large numbers may result in presslers. This can be counteracted by the use of either the "C<sub>2</sub>" or "H" culture together with *L. casei*. An active culture of *L. casei* can prevent this gas formation if the aerogenes organisms are only few in number.

Other organisms which bring about an overset condition in the cheese are being studied.

18. *The Use of Certain Streptococci as Starters for Swiss Cheese.* W. C. FRAZIER, L. A. BURKEY, K. J. MATHESON AND P. D. WATSON, Bureau of Dairy Industry, U. S. Dept. of Agriculture, Washington, D. C.

Certain streptococci which are found in many samples of raw milk and in freshly dipped Swiss cheese have been used as a starter for Swiss cheese in addition to the usual *Lactobacillus casei* (39a) starter. Two streptococci, "C<sub>2</sub>" and "H," which seem to be strains of *Streptococcus thermophilus* Orla-Jensen, have been used and a third streptococcus "C<sub>1</sub>," which has not been identified.

Experiments with the "C<sub>2</sub>" culture at Washington and at the Grove City, Pa. Creamery have shown that the proper use of this streptococcus in addition to the *L. casei* (39a) starter has been beneficial to the cheese by improving texture, eyes and flavor and consequently score, grade and selling price. It has also been possible to prevent the defects known as presslers and nisslers. If the "C<sub>2</sub>" starter and the *L. casei* are not used in the correct proportion glass and checking may result.

The "H" culture, although only a little different from the "C<sub>2</sub>" culture, is not as satisfactory for use as a starter. Results in Washington, Grove City and Ohio have indicated that there is a tendency toward oversetting when "H" is used. When difficulty has been experienced in obtaining eyes with the *L. casei* starter alone, however, good eye formation and good cheese have been obtained by the addition of

the "H" streptococcus. Cheeses have been made successfully in some cases with the use of the "H" starter alone.

The "C<sub>2</sub>" and "H" cultures when used with *L. casei* starter cause a considerably more rapid increase in hydrogen-ion concentration in the cheese in the press than the *L. casei* starter alone, although the final hydrogen-ion concentration is almost the same.

The "C<sub>1</sub>" streptococcus is very commonly found in raw milk and in Swiss cheese but apparently has little or no influence on acid development. A limited number of experiments has indicated that eye formation may be better in the presence of this organism.

19. *Observations on Butter Showing Surface Taint.* H. A. DERBY,  
Department of Dairy Industry, Iowa State College, Ames,  
Iowa.

Butter made from pasteurized cream inoculated with a small amount of surface taint butter and held for a number of hours before churning commonly developed the defect present in the inoculating material. A pronounced surface taint in the butter required from two to three days at 15.5°C. and from seven to ten days at 5.5°C. for its development. Experimentally produced surface taint butter also quite regularly caused the defect in butter made from cream into which it was inoculated. In this way the defect was sometimes produced down through a series of samples. Eventually, however, some other off flavor would overshadow the surface taint and become the prominent defect in the subsequent lots. Attempts to produce surface taint butter by the direct inoculation of a normal product with defective material were very largely unsuccessful.

An organism, which would produce typical surface taint butter, when inoculated into the cream from which the butter was made, was isolated from one sample of defective butter. The importance of this type under practical conditions is open to question because of the failure to find it in other samples. However, there are factors which may be of importance in this connection; e.g., the organism dies out rapidly in butter as judged by beef infusion agar plate counts.

20. *Notes on Monilia-like Organisms from a Trickling Filter Receiving Creamery Wastes.* H. E. GORSELINE WITH MAX LEVINE, En-  
gineering Experiment Station, Iowa State College, Ames, Iowa.

Monilia-like organisms were isolated from an experimental trickling filter receiving creamery wastes. Carbohydrate fermentations were not found to be particularly suitable for differentiation, but on the basis



of utilization of organic acids two types were recognized. The organisms are probably of significance in the elimination of inhibiting acidities from filters receiving carbohydrate wastes.

**21. The Bacteriostatic Effect of Gentian Violet on Thermophilic Spore-forming Bacteria.** E. J. CAMERON, National Canners Association, Washington, D. C.

As a group, the spore-forming thermophilic bacteria have been found to be highly sensitive to gentian violet. Most of the work in this laboratory has dealt with those types of thermophilic spore-formers which are of importance in the canning industry. The "flat sour" group, (facultative anaerobes), is definitely more sensitive than the group of spore-forming thermophilic anaerobes. This fact has been made use of in purifying mixed cultures of the two.

The effect of the dye has been tested on thirty-six strains of "flat sour" bacteria. On solid media, (yeast-water agar), and using the "streak" method of inoculation, all strains were completely inhibited in the 1:100,000 dilution on incubation at 55°C. At this temperature complete or partial inhibition was evident with all strains in dilutions up to 1:500,000. At 37°C., the bacteriostatic effect was more marked and a dilution of 1:1,000,000 completely inhibited growth in all cases.

In liquid media (glucose-yeast-water broth), the dye is more effective than when in solid media. Inhibition is usually complete in liquid media in a dilution of 1:750,000 and in many cases in a dilution of 1:1,000,000.

The age of the culture is a factor. Also it has been found that the spores are more sensitive than the vegetative forms.

**22. Lactic Acid Production of Some of the Anaerobes.** R. V. HUSSONG AND B. W. HAMMER, Dairy Industry Department, Iowa State College, Ames, Iowa.

During a study of the acids produced in milk by some of the anaerobic bacteria, the presence of lactic acid was investigated. The zinc salts of the non-volatile, ether-soluble acids were prepared. The data secured on these salts indicated that each of the five cultures used—three of *Clostridium welchii* and two belonging to the *Clostridium butyricum* group—produced lactic acid; the *C. welchii* cultures appeared to have produced lactic acid that was largely *d* or a mixture of *d* and inactive, while the *C. butyricum* cultures appeared to have produced inactive lactic acid.

**23. *The Microorganisms of Cabbage and Their Relation to Sauerkraut Production.*** C. H. KEIPPER AND E. B. FRED, College of Agriculture, University of Wisconsin, Madison, Wis.

The bacteriological and chemical changes which take place in the formation of sauerkraut are due to the microorganisms found on the plant tissue. The original flora naturally includes a great variety of microorganisms. The conditions, however, for the growth of these microorganisms in the kraut vat are such that the variety of the flora is rapidly reduced. Under normal conditions there is an enormous development of the lactic acid forming bacteria.

During the last two years, a bacteriological analysis has been made of 37 heads of cabbage. The average number and kinds of microorganisms on the outside and on the inside of representative heads of cabbage have been determined.

	<i>Total number of bacteria in 1 gram of tissue</i>
Outside of cabbage head, unwashed.....	2,000,000
Inside of cabbage head, unwashed . . . . .	4,000
Outside of cabbage head, washed . . . . .	800,000
Inside of cabbage head, washed.....	4,000

The results show an average of two million organisms for the unwashed heads, outside leaves removed. Ten-gram samples taken aseptically from the interior of various heads always showed the presence of microorganisms. The average number of organisms varied considerably, depending on the compactness of the heads. Young, open heads were found to contain a considerably higher number than the fully mature heads. The number of organisms on the outer leaves was greatly reduced by the process of washing.

A much higher percentage of a catalase-negative, small, pinpoint colony, presumably lactic acid forming organisms, were found on the interior of the heads than on the outside. Washing was tried in an effort to increase the percentage of the lactic acid forming bacteria. The results of tests in which 26 barrels each of washed and unwashed cabbage were made into kraut showed a superior quality of sauerkraut from the washed cabbage.

**24. *Studies in Microbial Thermogenesis. III. The Effect of Salting on the Microbial Heating of Alfalfa Hay.*** L. S. STUART AND L. H. JAMES, Food Research Division, Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington, D. C.

Laboratory heating experiments were conducted with moistened, salted and unsalted alfalfa hay. No heating was recorded under anaero-

bic conditions. Thirty per cent moisture was found to be the optimum moisture content for microbial heat production. The addition of 5 per cent salt failed to prevent microbial heating. However, salting was found to induce a considerable delay before heating started, depending upon the relation of the amount of salt to the total moisture. The addition of 2 per cent salt prevented bacterial growth, but failed to prevent mold development although the growth of the mold was considerably delayed. Results would indicate that the prevention of molding and "spontaneous" heating of hay by the addition of salt would only be efficient under such conditions as would allow for drying below the critical point for mold development within the short period of delay caused by the salt.

25. *A Contribution to the Bacteriology of Grass Silage.* KARL J. DEMETER, Bakteriologische Abteilung der südd. Forschungsanstalt für Milchwirtschaft in Weihenstephan, Bavaria, Germany.

The decided changes during the fermentation period of grass silage are brought about by a sporeforming, long rod in symbiosis with *B. subtilis*. Both organisms could be found in almost equal proportions. The characteristics of the sporeforming, long rod are summarized briefly as follows.

On all media containing traces of fermentable sugar it looks like a typical gram-positive *Lactobacillus* (length 3.4 to 4.3 $\mu$ , diameter 0.7 to 0.9 $\mu$ ). This is the reason why the ability of spore formation may be easily overlooked. This case suggests, that many other "Lactobacilli" might turn out to be real bacilli if cultured on suitable media! Spores are ellipsoid, almost terminal (length 1.8 $\mu$ , diameter 1.0 $\mu$ ).

Motility by peritrichiate flagellae. Growth good on artificial culture media containing fermentable sugar, otherwise scanty, streptococcus-like. No growth on gelatin. Optimum temperature 45°C., minimum 29°C., maximum 61°C. It ferments the following substances: glucose, maltose, sucrose, raffinose, dextrin, starch and glycerol. The acid produced is almost all *d*-lactic acid (91 per cent of the theoretical amount, analyzed by C. Neuberg), also some acetic acid and traces of other organic acids. In liquid sugar media there is production of an agreeable odor like that of the ether of malic acid, or fresh bread. Anaerobic conditions seem to promote growth in liquid media. Vigorous catalase production. Weak CO<sub>2</sub>-production from glucose and pepton. Best nitrogen source is pepton; asparagin may serve as source of both carbon and nitrogen. There is some ammonia production from pepton. No

production of indole or hydrogen sulphide. Nitrates are not reduced. Litmus milk is reduced with rennin-like coagulation. Potato: small yellow glistening layer.

Concerning *B. subtilis*, it was found that, under strictly anaerobic conditions, its activity results in carbohydrate fermentation and acid production (mostly non-volatile acid and some acetic acid). In mixed cultures with the above named lactobacillus-like rods there are indications that the rapid acid production of the latter undergoes some relaxation with tendency to suppression of any production of volatile acids, especially under anaerobic conditions. Two strains showed some destruction of xylose, when in mixed culture with *B. subtilis*.

26. *A Study of Pin-Spot Molding on Shell Eggs in Cold Storage.* L. H. JAMES AND T. L. SWENSON, Food Research Division, Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington, D. C.

In a study of a new type of mold-spoilage of shell eggs, which is confined largely to the exterior surfaces of the shells, two types of molds were isolated, a *Penicillium* and a *Cladisporium*. Eggs from California, Washington, and the Middle West were subjected to various treatments, inoculated with the *Cladisporium* and placed in the usual egg cold-storage. Different lots of the eggs were given the following treatments: Sweated, shipped in cases made of green or partially seasoned wood, with dampness in flats and fillers, washed, oil protected, and untreated (controls). Half of each lot was inoculated and then all the eggs placed in storage. Periodic examinations of the eggs showed that only the eggs stored in cases made of unseasoned wood developed the characteristic black colonies and then only to a negligible degree.

Eggs shipped from California to New York in cases of unseasoned wood showed definite pin-spot molding on arrival in New York. In subsequent cold-storage these eggs showed the development of the mold to a marked degree.

The foregoing results indicate that the development of the mold in cold-storage is dependent upon the previous existence of conditions which permit germination.

It is planned to conduct further work on this problem.

27. *Some Characteristics of Bacteria Causing Mustiness in Eggs.* MAX LEVINE AND D. Q. ANDERSON, Department of Bacteriology, Iowa State College, Ames, Iowa.

A number of organisms isolated from eggs which were rejected be-

cause of a musty odor were shown to be capable of producing mustiness in standard nutrient media (gelatin and agar) and in eggs. They were all found to be non-spore forming gram negative rods. Several types may be differentiated by gelatin liquefaction and carbohydrate or alcohol fermentations.

# ADVANCES IN THE STUDY OF STREPTOCOCCI<sup>1</sup>

LUDVIG HEKTOEN

*The John McCormick Institute for Infectious Diseases, Chicago*

A brief excursion into the past may be permitted in order to provide an historical background and continuity for considering certain recent advances in the study of the streptococci. In his discussion of the streptococcus problem, Dible traces the history of our knowledge of the streptococci in their classifications which he separates into three stages: the morphologic, the biologic and the immunologic. Undoubtedly, streptococci were seen in human lesions before they were distinguished from other bacteria by Ogston and others. The so-called colonies and masses of micrococci described by Klebs and by Rindfleisch and others in wound infections and pyemic lesions surely contained streptococci. It is probable too that the minute glistening specks that Virchow saw in thrombophlebitis and pyemia were in part streptococci. And Winge in 1869 had streptococci under his eyes when he described the microscopic organisms in the lesions of his historical case of mycotic endocarditis as threads carrying globular bodies like pearls on a string. This case by the way is the first case of mycotic or infectious endocarditis recognized as such. The modern study of infectious endocarditis consequently begins with the description of an instance of streptococcus endocarditis. In 1873 the pathologist, Orth, described and pictured micrococci in chains in the peritoneal exudate in puerperal fever. When Pasteur in 1879 made his dramatic announcement in the Academy of Medicine in Paris of finding chains of spherical granules in puerperal fever he referred to Orth's observations; at the same time Pasteur said that he had seen the same form of microbe in 1860 and that a little later he had ascribed to it ammoniacal fermentation of the urine. In the meantime Ogston

<sup>1</sup>Presidential address, delivered before the Society of American Bacteriologists at its thirty-first annual meeting, Ames, Iowa, December 30, 1929.

had described two forms of micrococci in suppurating wounds, one forming clusters, the other chains. To the latter Billroth in 1874 gave the name "streptococcus." The more exact study of streptococci now begins with the work of Fehleisen, Rosenbach, Passet and others, and the vibrios and micrococcal masses of the pathologists at the opening of the microbic era were resolved into components, of which streptococci and staphylococci were the most important. In 1883 Fehleisen isolated *Streptococcus erysipelatis* as the cause of erysipelas.

At first the possibility of more than one type of streptococcus was not considered. As the different infectious processes in which streptococci were found increased in number, the opinion developed that the streptococci included strains of different pathogenic powers. Marmorek and others, however, maintained that there is only one species of streptococcus pathogenic for man, the variations in its virulence accounting for the different forms of streptococcus infection. This streptococcus might cause now angina, now erysipelas, now septicemia, depending upon its state of virulence and the mode and place of infection. Put in another way—the streptococci are poorly differentiated but easily adaptable, and individual species cannot be recognized. Then came Schottmüller's significant separation of hemolytic from non-hemolytic streptococci according to their action on red corpuscles, subsequently made more precise and refined by the observations of J. Howard Brown. The attempts to separate streptococci of either hemolytic or nonhemolytic class into sharply defined pathogenic groups by their actions on sugars have not given decisive results of practical value.

The great question whether the nonhemolytic group contains the causative agents of distinct diseases is not an easy one to answer. The group appears to be a reservoir of diverse pathogenic possibilities. It contains strains causing local inflammation and focal infections and that it may contain also strains causing specific infectious diseases of course cannot be denied offhand. It is said that the only definite disease now established as caused by members of this group is subacute bacterial endocarditis. Green-producing streptococci are found as a rule in the endocardial lesion of the disease and endocarditis is produced with them

without difficulty in rabbits. All students of the causation of rheumatic fever are driven to concern themselves with the group of nonhemolytic streptococci and at present they form perhaps the most important elements in the most promising working hypothesis in that field. The intimate association of members of this group with epidemic poliomyelitis (Rosenow) and encephalitis (Rosenow, Evans) and with measles (Tunncliff) has been established but the precise nature of this relationship is not settled acceptably to all concerned in its investigation.

We now come to the question whether the hemolytic streptococci form a closed group in which there are no constituents with distinct pathogenic properties. The unitarian view of hemolytic streptococci seemed to have been upset more or less completely by the establishment of immunologic groups, of which at least two were associated with definite diseases—scarlet fever (Moser and von Pirquet, Ruediger, Tunncliff, Bliss) and erysipelas (Tunncliff). This is the immunologic stage in Dible's classification, but it is to an independent departure, namely the study of toxins as the bearers of specific pathogenic powers on the part of streptococci, that I wish to give special attention. As I shall limit my subsequent discussion to questions relating to streptococcal toxins, references may not be made to much of recent, important work on scarlet fever and related problems.

In discussing in a general way a subject that is under such active scrutiny as the toxigenic power of certain streptococci is at present, it cannot be expected that final conclusions will be reached. But the effort may indicate the trends of development and significant relations. In order to maintain a definite object in view I shall consider the situation as if endeavoring to find out for myself whether or not certain streptococci can produce toxins and whether or not there is any good reason to doubt that specific forms of streptococci cause scarlet fever, erysipelas and perhaps other diseases.

Turning first to the streptococcus of scarlet fever, let me recall that the old question whether scarlet fever can be caused by streptococci appears to be answered affirmatively in the following experiments by George F. Dick and Gladys Henry Dick: The



nurse of a patient with scarlet fever came down with a mild but typical attack of the disease. Two days before she noticed that she had a sore finger. On the second day, when the rash was intense, a few drops of pus were obtained from the finger and from this was grown a hemolytic streptococcus, cultures of which were applied by swabbing to the tonsils of five persons, and in one of them, a woman aged twenty-five years, who was inoculated when the streptococcus had grown on artificial mediums for three weeks, there developed a typical, mild scarlet fever. The sterile filtrate (Berkefeld V) was inoculated without effect in five volunteers, one of whom, a woman aged twenty-three, later developed a typical scarlet fever, forty-eight hours after the application to the throat of an unfiltered culture of the streptococcus. This particular streptococcus fermented mannitol. Inoculated in the same manner, a streptococcus from scarlet fever not fermenting mannitol produced scarlet fever in a woman aged twenty-two. From these experiments it was concluded that hemolytic streptococci may cause scarlet fever.

Nicolle, Conseil and Durand also report the production of scarlet fever in many by introducing cultures of streptococci from scarlet fever into the throat. And Moriwaki says that he has produced scarlet fever in volunteers by inoculating into the throat scarlatinal hemolytic streptococci.

In the experiments of Doctor and Mrs. Dick with the streptococcus that fermented mannitol, ten young adults who said that they had not had scarlet fever were inoculated with this strain, but only two acquired the typical disease. This result naturally suggested that there are differences in susceptibility to scarlet fever. According to many observations, hardly one half of those exposed naturally develop the disease in typical form. The study of the skin reactions to the streptococcus used in these experiments showed that the filtrate in proper dilution caused a definite reaction in 41.6 per cent of persons who gave no history of scarlet fever, while in convalescents from scarlet fever there was no reaction or only a slight reaction; further, that all action of the filtrate on the skin was suspended either by mixing the filtrate before it was injected with the serum from scarlet

fever convalescents or by injecting such serum intramuscularly before making the skin test. Later it was found that the serum of horses immunized with the filtrate also neutralized its action on the skin of a person susceptible to scarlet fever. It was also found that while the skin test was positive before a person passed through scarlet fever, it became negative after the attack. This is the basis of the Dick test, now accepted generally as an index of resistance or susceptibility to scarlet fever. The discovery of this test in turn led to the idea that possibly the streptococci of scarlet fever produce a specific toxic substance or substances with which an active immunity may be produced. Accordingly experiments were made which showed that when susceptible persons are injected with the proper quantities of toxic filtrate, they may develop a scarlatinal rash and suffer nausea, vomiting, fever, and general malaise but not tonsillitis. It seemed as if "the unknown scarlet fever poison" of earlier days had been found. These phenomena, since observed by others, develop within a few hours of the injection and disappear in a short time. Following this reaction, the skin test, previously positive, became negative or merely faintly positive; in other words a degree of active immunity to scarlet fever can be produced, which is demonstrated also by the neutralization of the toxic effects of an active filtrate by the serum of a person who has received injections of the filtrate.

It seemed reasonable then to regard this toxic substance as a true toxin in the strict sense, hence capable of inciting the production of specific antitoxin. This proved to be the case, as horses injected with increasing quantities of the filtrate develop specific antitoxic substances that can be concentrated by the methods used in concentrating antidiphtheria serum. If given early in the attack, potent antitoxic serum against scarlet fever shortens the course of the disease and reduces the complications and sequelae. The serum also has a preventive effect of brief duration.

Of great importance is the demonstration, now abundantly substantiated, that scarlet fever toxin may be injected with safety in susceptible persons in quantities large enough to develop complete active immunity as determined by the development of a negative Dick test and freedom from scarlet fever.

Finally, by testing the power to produce toxin it may be shown whether a given strain of hemolytic streptococci can cause scarlet fever.

To Doctor and Mrs. Dick goes the merit of showing in what seems conclusive fashion the relation of certain streptococci and their toxin to scarlet fever. They opened fully the door towards which others may be said to have pointed.

I have restated familiar details in order to provide the background for certain comments. But first I wish to remind you that Birkhaug has reported results of work on the streptococcus of erysipelas which place that organism in the same relation to erysipelas as that in which the Dick work places the scarlatinal streptococcus to scarlet fever. In the work on erysipelas experiments on animals have yielded significant results that should not be left out in the consideration of the specificity of scarlatinal and erysipelas toxins and its practical significance.

It may be true that many of the workers who have concerned themselves with repeating and extending the investigations on the streptococci of scarlet fever and erysipelas have not accepted the view that they are specific pathogenic agents by virtue of specific toxigenic powers. At the same time it should be said that of course no one doubts the streptococcal nature of erysipelas and only few doubt the streptococcal nature of scarlet fever. The value of the Dick test, of preventive inoculation with scarlatinal toxin, and of antitoxic serum in erysipelas and scarlet fever may be doubted, however, for various reasons that need not be discussed at this time. The main concern now is to inquire whether the underlying principles of this departure are sound. It may be pointed out, however, that a great deal of work on scarlet fever and other streptococci and their supposed toxins must be discarded because of faulty technical methods. Numberless tests have been made with unreliable and unstandardized toxins and antitoxic serums. It is no wonder that skin tests with streptococcus filtrates and efforts at neutralization with homologous and heterologous antisera have yielded confusing results. In many such tests the question of the purely toxic, the allergic or the mixed nature of the reactions has not been taken into ac-

count. The nature of the streptococcal cultures employed has sometimes been taken too much for granted and various possibilities, for instance, that of working with mixed cultures, have not been considered, to say nothing about dissociation. It has been claimed too that toxic filtrates may contain more than one toxin and that a given person may be susceptible to one component and not to others. This view, which seems sound enough in theory, has found support in the observation that a given scarlet fever convalescent serum seemed to neutralize the toxin of a streptococcus in some susceptibles but not in others. Yet such a result may be due to quantitative rather than qualitative differences in susceptibility. There is of course great need for a fairly simple method of distinguishing the particular scarlet fever or erysipelas streptococcus, if such exist, from other streptococci. At present the opsonic test seems to be giving helpful results (Tunncliffe).

The view that the streptococci of scarlet fever and erysipelas are specific agents because they produce specific toxins is opposed mainly on two grounds:

1. The effect ascribed to the so-called toxin is not a direct toxic action but is in nature allergic.

2. Pathogenic hemolytic streptococci produce toxins that are more or less identic.

1. *Allergy in scarlet fever and erysipelas.* In the chronic and recurrent infections specific allergy may be an important factor. And in certain acute infections, particularly those with a rather long incubation period like smallpox and measles, general allergy also undoubtedly plays a part. During the incubation period, at the beginning of which infection takes place, the body responds by cellular changes with the production of specific antibodies, and at a given time there appears to occur a sharp reaction between the infecting organism, the antigen, and the cells of the body as well as the newly formed antibodies, which manifests itself in the symptoms and lesions of the disease. When the acute attack comes, the reactivity of the body is different from its reactivity at the time the infection took place—the body is now sensitized. The gradual development of this allergic state can be followed very closely in vaccination against smallpox. In 1802

Bryce demonstrated with startling clearness the hastening of the reaction at the site of each daily revaccination after primary vaccination, so that in each case the final stage was reached at the same time as in the primary lesion. A hundred years later Pirquet, without any knowledge of Bryce's beautiful demonstration, repeated the experiment with the same result. The long but definite incubation period in measles and in chickenpox suggests that here also the acute attack results from the reaction between the virus of the disease and the allergic patient. It may well be that somewhat similar relations exist in acute infections with a less precise incubation period such as erysipelas, diphtheria, scarlet fever. At any rate, the typical attack is associated with a wave like rise of antibacterial or anti-invasive antibodies in the blood which reaches the high point at the time of crisis. In these three diseases, however, there enters the question of the production of specific toxins capable of immediate and direct pathogenic action in the susceptible person. At present we are not concerned directly with diphtheria—there is no question about the primarily toxic nature of that disease.

Turning to streptococci we find that they may set up allergic states in human infections as well as under experimental conditions. In rheumatic fever the idea that streptococcal allergy is an important factor is receiving very earnest attention. On the allergic hypothesis a primary streptococcal focus is regarded as so sensitizing the tissue that streptococcal invasion now results in reactions that manifest themselves as some form of rheumatic fever. Appropriate experiments have been made on rabbits with confirmatory results (Swift). In rheumatic fever there commonly has existed for some time chronic and repeated streptococcal infections of the tonsils and the respiratory tract. Naturally the question of the value of skin tests in demonstrating rheumatic allergy arises. At the moment there seems to be no way of distinguishing cutaneous sensitiveness due to streptococcal infections that may have nothing to do with rheumatic fever from the cutaneous sensitiveness due to focal streptococcal infections that may lead to rheumatic fever. Nevertheless the skin test may prove of value if it is established that a positive reaction occurs in

a high percentage of active and inactive cases of rheumatic fever (Birkhaug). While the statements about rheumatic allergy apply mainly perhaps to the action of nonhemolytic streptococci, they apply also to the hemolytic streptococci. It has been found that hemolytic streptococci from tonsils, adenoids, and paranasal sinuses of patients suffering from subacute glomerular nephritis may produce toxic filtrates that cause cutaneous reactions, sometimes intense, especially in patients having nephritis associated with streptococcal infection (Longcope), and thus suggesting a connection. It is true that the question whether such filtrates are truly toxic or act by way of allergy is under discussion, but it may be accepted that at least part of their action is allergic. The question arises whether there are any detectable differences in the cutaneous reactions caused by primary toxic actions and those of allergy. In all work of this kind it must not be overlooked that a bacterial exotoxin may be associated with allergenic substances, e.g., from the bacteria, from the medium, from unexpected contaminations. I have found that hemolytic streptococci grown on a medium containing beef or sheep protein may retain such protein after repeated careful washing in salt solution. On injecting into rabbits crude extracts obtained by crushing such streptococci, precipitins developed in large amounts for the animal protein in the culture medium. Bacterial extracts and filtrates from suspicious sources might be tested with advantage for the presence of foreign antigens before they are used in investigative work on delicate immune reactions.

It must be accepted, however, that pure streptococcal allergy may exist, and on account of similarities in the constituents of streptococci there may be more or less overlap between possibly different strains in strictly allergic streptococcal reactions. Consequently it cannot be denied that reactions to the Dick toxin or to erysipelas toxin, both cultural filtrates, may be to a greater or less extent due to allergy from previous streptococcal infections. And the view that some of the complications of scarlet fever may be allergic in nature does not seem unreasonable. Direct tests could be made to determine whether there is any true streptococcal allergy after the attack.

Scarlet fever itself has been claimed to be an allergic disease, at first of unknown origin, more recently of streptococcal origin (Bristol). In either case the incubation period, the rash, the eosinophilia, the swelling of the lymph nodes, and even the immunity have been held to support the allergic hypothesis. On the streptococcal hypothesis scarlet fever as a whole is regarded as an allergic or hypersensitive reaction to streptococcal protein followed by a more or less permanent desensitization and immunity. The allergy is ascribed to streptococcus infection of some sort early in life. Without necessarily subscribing to this comprehensive hypothesis, many put forward the view that the Dick reaction is wholly allergic, the allergy dating probably from an early period. The disappearance of the reaction has been explained as due to desensitization and also to the development or introduction of antitoxin. But such views are really not in full harmony with our knowledge of allergy. Desensitization as a rule is not permanent; in most cases that effect is temporary only. Furthermore, the active substance or substances that directly cause allergic or anaphylactic reactions are not true toxins with antigenic properties and neutralizable by specific antitoxin. The Dick toxin is neutralized by antitoxin; it is antitoxigenic; on immunization with it, precisely the same sort of results are obtained as on immunization with diphtheria toxin. In scarlet fever the blood and urine contain a toxic substance that causes a skin reaction in persons whose serum does not blanch the scarlatinal rash, that is, is not antitoxic, but not in persons whose serum blanches the rash, that is, is antitoxic (Blake). This substance is neutralized by antiscarlatinal serum and clearly appears to be the same as the substance that causes the scarlet rash and as the toxin in the filtrates of scarlatinal streptococci; in the course of scarlet fever the serum acquires the power to blanch the scarlatinal rash, but this power may not be developed fully until the tenth day (Birkhaug). These facts appear to me to favor the view that scarlet fever is mainly a toxic disease, that it is caused by a streptococcus or streptococci which produce specific toxins and that recovery is associated with the production of antitoxin. The immunity in scarlet fever is basically antitoxic;

if it has been made complex in theory, it is simple enough in practice because it is effective and, at least in the natural form, permanent.

Since 1924, pupil nurses about to enter the hospital of the John McCormick Institute for Infectious Diseases have been tested for susceptibility to scarlet fever. Most of the susceptibles have been immunized to negative skin test before beginning their work. Of 203 originally insusceptible nurses and of 112 susceptible nurses completely immunized before entrance, not a single one has developed scarlet fever in the hospital. Before 1924, 7.7 per cent of all pupil nurses in the hospital came down with scarlet fever. By sorting children and making the susceptible resistant we can control scarlet fever. The difficulty in the way of attaining this result appears to be mainly administrative in nature.

The allergic theory has been used to explain certain rare cases of recurrent erysipelas in which the process starts some distance away from the focus of infection. The assumption is that streptococcal material is absorbed at the focus of infection and carried to areas that were made allergic in previous attacks. The question arises how to exclude completely the presence of the streptococcus of erysipelas from within the lymph vessels of the area involved. Also, might not absorbed toxin be responsible for the lesion?

It is claimed, too, that primary erysipelas is not due to the production of specific toxin and that recovery consequently does not depend on the development of specific antitoxin but on allergy to streptococcal products in the erysipelatous area. That the disease is wholly allergic (Francis) is not tenable in view of the susceptibility of the newborn to erysipelas unless, indeed, intrauterine streptococcal sensitization can be established. While allergic and anti-infectious processes may play a part in erysipelas, nothing has been brought forward that excludes the action of specific toxin neutralizable by the corresponding antitoxin. Here also blanching of the redness has been produced by the injection of antitoxic serum and a toxic substance identical with the toxin in the streptococcus culture has been demonstrated in the urine and the blood of erysipelas patients (Birkhaug).



In connection with this question of the possible allergic nature of supposedly toxic streptococcal filtrates Ando's recent work is of much interest. He isolated from Dick toxin a nucleoprotein and a specific toxin, both of which caused typical skin reactions. The toxin is destroyed by heating at 80°C. for 30 minutes and is neutralized by antitoxin; susceptible persons may be immunized with it and in some instances the scarlatinoid syndrome has developed. While no evidence of allergy was obtained with the toxin, the nucleoprotein proved to be allergenic. The protein may influence the reaction to the Dick toxin as ordinarily prepared, especially if it is weak in true toxin. Ando found the toxin prepared by the Hygienic Laboratory in Washington to contain only traces of nucleoprotein, and also that it is desirable always to use toxins that are potent in high dilutions. If confirmed, these results may remove all doubts as to the presence of true and specific toxin in streptococcal filtrates.

2. *Pathogenic hemolytic streptococci produce toxins that are more or less identic.* It is reported that hemolytic streptococci from scarlet fever, from erysipelas, from puerperal infection and from other sources appear to produce toxin or toxins that are more or closely related if not identic. "In the majority of the cases of any disease caused by hemolytic streptococci, any part of the lesions due to exotoxins may be helped by any antitoxin." It is even claimed that one and the same toxin is at work in all hemolytic streptococcus infections, which accordingly should be treated preventively as well as curatively with just one antitoxic serum. These claims provoke the question, Are the streptococci concerned in scarlet fever, erysipelas, septic sore throat, and other streptococcal infections specific streptococci, or do they have in common essential antigenic and biologic powers? The surgeon operates upon a patient with acute appendicitis and peritonitis. He punctures the skin of his finger with the point of a needle. In a few hours grave general symptoms develop. Death takes place in two or three days from general streptococcus infection, with but slight reaction at the point of puncture. Accepting without any question that cases of fulminant streptococcus infection do occur, what is the nature of the substances that can cause such

overwhelming effects? Are they the same as the toxins of scarlet fever and erysipelas? Is hemolytic streptococcus gangrene, which is regarded as a clinical entity, due to a specific streptococcus? Do streptococci from puerperal fever have special toxigenic (Lash and Kaplan) powers? And is *Streptococcus epidemicus* (Davis) a homogeneous strain with peculiar pathogenic properties? Are pathogenic, hemolytic streptococci, in fact, one species with common pathogenic potentialities, and are we simply grinding over the old grist of our fathers? Perhaps a brief consideration of some phases of scarlet fever and erysipelas may throw light on such questions.

Owing to its marked characteristics, erysipelas has been recognized as a distinct disease since Hippocratic time. It has never been confused with scarlet fever. The two diseases have been regarded as distinct since scarlet fever was recognized. The epidemics of the two diseases have been distinct. There are no records of epidemics in which some of the patients had scarlet fever and some erysipelas or in which several patients had both these diseases at the same time. In fact there are remarkably few instances in which erysipelas has complicated scarlet fever, or vice-versa, as would be expected to be a not unusual occurrence if both these diseases were caused by the same agent and the same toxins. The only instance I have found to be put forward as supporting the identity of the two diseases is that of a physician who contracted a typical case of facial erysipelas while treating a patient with scarlatinal sore throat. Of 3979 cases of scarlet fever treated in the Durand Hospital of the John McCormick Institute for Infectious Diseases, only three developed erysipelas, all of the face, and in one of these cases it seems doubtful whether erysipelas was present. Of 48,366 cases of scarlet fever in the Metropolitan Asylum Board Hospitals complicated by some other disease, in 1094 cases the complicating infection was diphtheria, in 899 cases chickenpox, in 703 measles, in 404 whooping cough, in 55 erysipelas (or 1 in 880), in 11 typhoid fever, and in 1 typhus fever. There is less conjunction of scarlet fever with erysipelas than with diphtheria, measles and whooping cough. Scarlet fever was introduced into the Faroe Islands in 1873 and became

epidemic there until 1875. For fifty-seven years previously the islands had been free from the disease. During the 1873-1875 epidemic 38.3 per cent of the population suffered from clinically recognized scarlet fever, but there is no mention of any outbreaks of erysipelas during this epidemic as would be expected to occur if erysipelas and scarlet fever are due to the same streptococcus. From an analysis of 20 cases of erysipelas and 981 cases of scarlet fever over a course of ten years at the Children's Clinic in the Berlin Charitè, Borkevitch concludes that erysipelas is totally distinct from scarlet fever in its clinical course.

If the streptococcus that causes scarlet fever is the same as the one that causes erysipelas, how are we to explain that, following scarlet fever, permanent immunity is established while, following erysipelas, the immunity is transitory and subsequent attacks are not infrequent? And how explain that scarlet fever is communicated to animals only with great difficulty while erysipelas is communicable to animals more easily? The relation of erysipelas to scarlet fever in the natural history of these diseases is precisely that of two causally distinct diseases. The streptococci concerned in scarlet fever and the streptococci concerned in erysipelas consequently are pathogenically as different as the meningococcus is different from the gonococcus and scarlet fever is as distinct from erysipelas as epidemic meningitis is distinct from gonococcus infection. Moriwaki reports the results of experiments in which he found it impossible to distinguish the streptococci from cases of scarlet fever from other streptococci by any available method, including toxin production and neutralization by antiserum. Finally he resorted to the study of the production of a scarlatiniform rash by the various streptococci, with the result that such rash was produced by all his scarlatinal strains but not by any of his other strains except one of the nine erysipelas strains he tested. In the discussion, Moriwaki clearly undervalues the significance of this result, which after all is in harmony with Langowoi's observation in 1906, who saw no rash in twenty children who received a vaccine of streptococci from erysipelas, while 13.3 per cent of 120 children who received vaccine

of scarlatinal streptococci developed rash followed by desquamation. Gabritschewsky emphasized that vaccine of erysipelas streptococci did not protect against scarlet fever. Please note also that there is no evidence, epidemiologic or other, that scarlet fever arises, so to speak, *de novo* from other sources than previous cases of the disease, as would be expected if the hemolytic streptococci are pathogenically equivalent.

Let us now consider the results of skin tests with the toxins of scarlet fever and erysipelas and of their cross neutralizations with the respective antisera. Birkhaug tested 407 children and adults and obtained a positive reaction with the scarlatinal toxin in 52 per cent and with the erysipelas toxin in 24 per cent, but only in 10 per cent with both toxins. These results have been confirmed and extended by Doctor and Mrs. Dick, using carefully standardized preparations in all the tests. Of 500 persons of all ages 36 per cent gave positive reactions to the scarlet fever toxin and 46.4 per cent reacted positively to the erysipelas toxin; Of those immune to scarlet fever 42.1 per cent were susceptible to the erysipelas toxin, and of those immune to erysipelas 31.3 per cent were susceptible to scarlet fever toxin. Of 100 convalescents from scarlet fever, the skin reaction to the scarlatinal toxin having become negative, 52 per cent gave positive reactions to the erysipelas toxin. Next, 24 persons susceptible to both toxins were immunized with the scarlet fever toxin, and it was found that while the skin no longer reacted to this toxin, the reaction in all cases was positive to the erysipelas toxin. In neutralization tests the standardized sera of horses immunized with the toxin produced by a single strain of either scarlet fever or erysipelas toxin was used. The tests were conducted on persons susceptible to both toxins and each toxin was neutralized only by its own antiserum. It was shown also that these antisera neutralized specifically the toxins of a number of other strains of streptococci and in the course of these tests it appeared that erysipelas streptococci produce considerably weaker toxin than scarlet fever streptococci, which may account in part for the rather weak immunity following an attack of erysipelas.

These results indicate clearly that there is no relationship between the toxins of the streptococcus of scarlet fever and the toxins of the streptococcus of erysipelas, and that these streptococci are pathogenetically distinct and produce specific toxins.

I conclude that etiologically erysipelas and scarlet fever are as distinct and different as they are clinically. The demonstration of the specific toxigenic power in streptococci, the latest great advance in their study, justifies the hope that further differentiation in streptococcal pathogenesis can be accomplished with further results of practical value.

# THE EFFECT OF AUTOLYSIS IN STERILE TISSUES ON SUBSEQUENT BACTERIAL DECOMPOSITION

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In an investigation on the rates of autolytic and bacterial decomposition of haddock muscle, Reed, Rice and Sinclair (1929) observed that when bacteria act along with autolytic enzymes "the content of non-coagulable nitrogen and ammonia nitrogen increases during the first 24 hours only slightly more than under the influence of the tissue enzymes alone; but during the second 24 hours of the combined tissue enzyme and bacterial action the rate of protein transformation increases enormously over the action of the tissue enzymes alone."

Falk and McGuire (1919) state that if meat is kept cold, allowing autolysis to proceed, and then is brought to room temperature decomposition will be much more rapid because of the simpler products formed by autolysis which would serve as nutriment for bacteria. Browne (1918) came to the conclusion that autolysis rather than bacterial action plays the important part in the initial stages of decomposition of fish stored on ice.

Bainbridge (1911) and Rettger and his co-workers (1915, 1916, 1918) have shown that bacteria are unable to attack native proteins and that the proteoses and higher polypeptides are also resistant to the direct attack of organisms. Robinson and Rettger (1918) found that bacteria do not require proteins, even in minute quantities, to develop normally, but obtain their sustenance from the amino acids and perhaps some of the simpler polypeptides. We might, therefore, expect some change in the

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proteins necessary before the organisms can find a suitable substratum. More recently, Hucker and Carpenter (1927) have shown that the amount of nitrogen available for bacterial growth varies considerably during the digestion of proteins when determined by the minimum amount necessary to produce visible growth of certain test organisms. They state that there appears to be no relation between the amount of amino nitrogen and the ability of partly digested proteins to sustain bacterial growth.

Cruickshank (1911) investigating the histological changes in autolyzing kidney found that *Staph. aureus* introduced into kidney that had autolyzed 24 hours at 37°C., produced very little change in the first 24 hours, although there was active multiplication. During the next 24 hours, however, there was a rapid softening and cellular change. On the other hand, these organisms introduced into fresh kidney produced a picture of complete necrosis in 10 to 15 hours, as compared to 12 to 15 days required by autolytic enzymes. This may be explained by the fact that the organisms first use up the simpler products of autolysis before attacking the more complex substances. Rice (1927) found this to be the case when organisms were introduced into clam muscle previously autolyzed under toluol and then autoclaved to sterilize and drive off the toluol. At first there was a decrease in non-coagulable nitrogen, that is the simpler products of autolysis were being utilized.

With these considerations in mind, the following problem was undertaken. The early part of the work was done on haddock muscle. For the latter part, guinea pig kidney was used. These were collected aseptically and aseptic autolysis compared with the effects of organisms introduced at various stages of autolysis.

#### EXPERIMENTAL METHODS

##### *1. Collection of aseptic haddock muscle*

All the fish used were caught on trawls and brought to the laboratory alive. They were then kept in large concrete tanks in a cool dark basement. Fish have been kept alive in these tanks for two months in good condition. The fish was stunned

by a blow on the head and inverted in a narrow box to hold it in position. Two narrow strips were then seared across the fish with a gasoline blow torch, from a point just posterior to the anus to the middle of the second dorsal fin, and from the interspace between the first and second ventral fins to a corresponding point between the second and third dorsal fins. This effectively charred the skin, but the heat did not penetrate to any extent into the flesh. Two incisions were then made across these

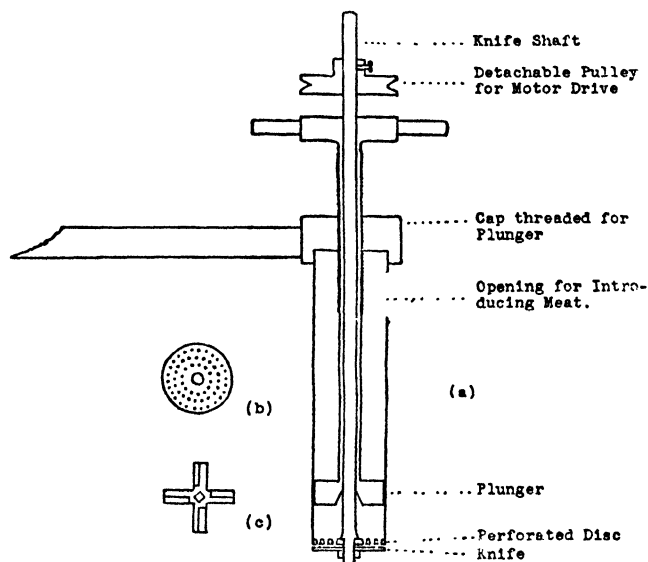


FIG. 1. *a*, CROSS-SECTION OF MEAT CHOPPER; *b*, PERFORATED DISC; *c*, REVOLVING KNIFE

charred bands, cutting to the spinal column. The tail was deflected to one side and cylinders of muscle taken out from the portion between incisions with sharpened steel "cork borers."

To ensure grinding the meat finely enough to give an even suspension and at the same time keeping the material sterile, a special meat chopper was devised (fig. 1). All instruments were sterilized by heat, i.e., kept at 170°C. for one hour. The meat was removed as described above, transferred to the meat chopper and ground into bottles of sterile saline. The result was a



fairly even suspension of muscle shreds which could be pipetted quite readily. The 24 and 48 hour determinations were made on samples prepared in this manner which had been incubated at 25°C.

Two or 3 grams of muscle were ground into 20 cc. of saline in a small wide mouthed bottle and a bottle used for each set of determinations. A given number of bottles were prepared, depending on the length of the series under observation. Being all from a restricted area and from the same side of the fish, each series should be fairly uniform.

### *2. Collection of sterile guinea pig kidneys*

The pig was killed by neck stroke, the skin of the abdomen dissected back and the flesh seared. The kidneys were then removed with aseptic precautions and each one ground, as in the case of the fish muscle, into a bottle containing 60 to 70 cc. of saline. The bottles were incubated at 37°C. Sterility was checked throughout by smears and by culture at the end of a series.

### *3. Chemical methods*

To obviate weighing and minimize chances of contamination, all results are calculated as percentage of total nitrogen for that particular sample. Owing to the small amounts used, micro-methods were resorted to throughout. Analyses were made for total, non-coagulable and ammonia nitrogen on the fresh tissue and at varying periods of incubation.

*a. Total nitrogen.* The method used was a combination of the methods of Folin and Denis (1916) and Koch and McMeekin (1924). The digestion mixture used was one volume of concentrated sulphuric acid and three volumes of concentrated (85 per cent) phosphoric acid. One cubic centimeter of this was used for 1 cc. of fish or kidney suspension.

As soon as the dense acid fumes filled the tube, 30 per cent hydrogen peroxide (Merck's Superoxal) was added drop by drop until on further heating the mixture remained colorless. Heating was then continued for five minutes. On cooling, water was

added to the 50 cc. mark. Twenty-five cubic centimeters were taken for a determination and Nesslerized in the usual manner.

*b. Non-coagulable nitrogen.* The proteins were precipitated with 5 per cent trichloroacetic acid, 5 cc. being added to 5 cc. of the tissue suspension. This was allowed to stand one half hour at room temperature and filtered. Five cubic centimeters of the filtrate were then digested as for total nitrogen, using a diluted digestion mixture (1:1). Fish muscle digests could not be Nesslerized directly as the solution invariably clouded. The ammonia was aspirated into dilute acid and then Nesslerized. This difficulty was not encountered in kidney digests and they were Nesslerized directly.

*c. Ammonia nitrogen.* In determinations on fish muscle, the trichloroacetic acid filtrate was made alkaline and the ammonia aspirated into dilute acid. With the kidney the method of Sumner (1918) was used; 5 cc. of  $\text{CuSO}_4$  solution (298 grams per liter) were added to 10 cc. of the suspension. Five cubic centimeters of 10 per cent  $\text{NaOH}$  were added, the mixture shaken and then filtered. Ten cubic centimeters of the filtrate were used for each determination, and Nesslerization carried out in the usual way. Owing to the small amounts of ammonia met with, comparisons often had to be made with the naked eye, using a range of standards.

#### *4. Determination of non-coagulable nitrogen in aseptically collected fish muscle*

During the early part of the season most of the samples were sterile. However, at this time experiments were still in progress as to a simple and satisfactory method of determining the non-coagulable and ammonia nitrogen. The tables, then, include only a few determinations made near the last of the summer, and at this time many of the samples taken were contaminated with a large Gram-positive, spore-bearing bacillus. It will be seen (tables 1 and 2) that about 59 per cent of the samples taken were sterile. The determinations on the contaminated samples are included in the tables but are not considered in the averages. It

should be noted that the number of organisms found in the contaminated samples was very small.

TABLE 1

*Percentage of non-coagulable nitrogen in fresh haddock muscle and in aseptically collected muscle after autolysing 24 and 48 hours*

Contaminated samples marked "x" and not included in the average

SERIES	PER CENT OF TOTAL NITROGEN		
	Fresh	After 24 hours	After 48 hours
A	8.14		
	7.46		
B	7.94		11.35
C	9.38	9.90	11.16
			20.20x
D	8.80	6.72x	14.38x
		6.86x	
E	9.13		
G	9.70	9.60	11.50
Average.....	8.65	9.75	11.33

TABLE 2

*Percentage of ammonia nitrogen in fresh haddock muscle and in aseptically collected muscle after autolysing 24 and 48 hours*

Contaminated samples marked "x" not included in average

SERIES	PER CENT OF TOTAL AMMONIA NITROGEN		
	Fresh	After 24 hours	After 48 hours
B	0.25	0.72	0.92
C	0.48	0.59x	
D	0.34	0.36x	0.47x
E	0.25	0.26	
F	0.47	0.84	0.67
G	0.13	0.14	0.37
Average.....	0.32	0.49	0.65

The percentage of non-coagulable nitrogen in the fresh samples, shown in table 1 and plotted in figure 2, is fairly constant. Occasionally a fish is found with a much higher non-protein nitrogen content (see table 3). This, however, is not the rule and is probably due to the condition of the animal at the time. There

is a slight increase in non-coagulable nitrogen at 24 hours, followed by a proportional increase at 48 hours.

5. *Determination of ammonia nitrogen in aseptically collected haddock muscle*

The ammonia nitrogen (table 2) shows a steady increase at 24 and 48 hours. At 48 hours this increase is 100 per cent, as compared with a 30 per cent increase in non-coagulable nitrogen.

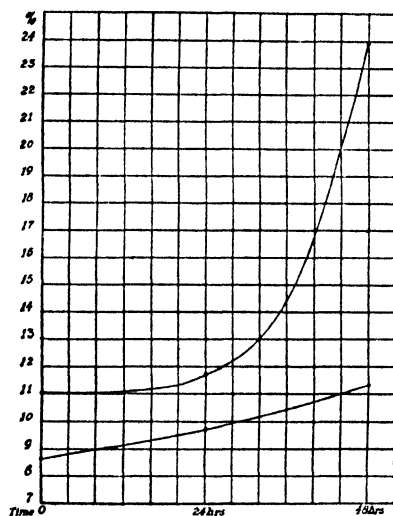


FIG. 2

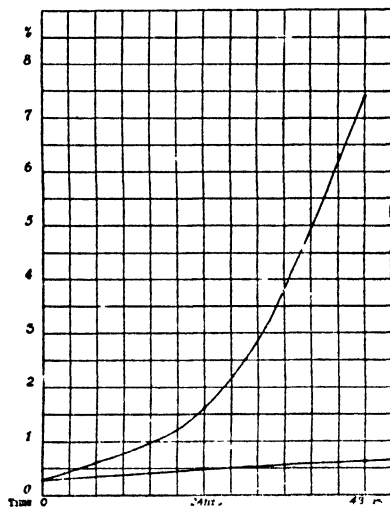


FIG 3

FIG. 2. INCREASE IN NON-COAGULABLE NITROGEN IN HADDOCK MUSCLE  
Lower curve: Sterile muscle. Upper curve. Infected muscle

FIG. 3. INCREASE IN AMMONIA NITROGEN IN HADDOCK MUSCLE  
Lower curve: Sterile muscle. Upper curve: Infected muscle

It will be noted (table 5) that the results obtained vary considerably from those of other workers. The decided increase which Reed et.al. (1929) obtained, was not found. While slightly higher, the results are of the same order as those of Sanderson (1926). It should be noted, however, that Reed was aerating the muscle filtrate directly, while Sanderson, following the proposal of Sumner (1918), precipitated the proteins with copper sulphate and then Nesslerized. This method is then very similar

to the one used here and is the one used in the latter part of the investigation.

#### 6. Determination of non-coagulable nitrogen in infected haddock muscle

To compare the above results with material undergoing both autolytic and bacterial decomposition, a few determinations were

TABLE 3

*Percentage of non-coagulable nitrogen in fresh haddock muscle and infected muscle after 24 and 48 hours*

SERIES	PER CENT OF TOTAL NITROGEN		
	Fresh	After 24 hours	After 48 hours
H	8.3	9.8	19.3
J	13.8	13.6	28.7
Average.....	11.05	11.7	24.0

TABLE 4

*Percentage of ammonia nitrogen in fresh haddock muscle and in infected muscle after 24 and 48 hours*

SERIES	PER CENT OF TOTAL NITROGEN		
	Fresh	After 24 hours	After 48 hours
H	0.37	1.9	5.9
J	0.13	1.3	9.1
Average.....	0.25	1.6	7.5

made on infected tissues. The fish was stunned, rapidly filleted and several samples taken from the same region used in the former determinations. No attempt was made to work with pure cultures in these early experiments. The material was infected with fresh haddock slime, the flora of which has been described by Reed and Spence (1929), and put through the meat chopper. Table 3 and figure 2 show that there is little change during the first 24 hours, followed by a very decided increase during the second 24 hours.

### 7. Determination of ammonia nitrogen in infected haddock muscle

Under the combined action of the autolytic and bacterial enzymes the increase in ammonia is enormous (table 4). Here again the results are of the same order as Sanderson's, although the increase in 24 hours is much greater than he obtained. The pH of the 48 hour samples rose to 7 or over, in spite of the buffers, so that even this large amount may not represent all of the ammonia produced.

### 8. Determination of non-coagulable nitrogen in sterile kidney

Kidneys were collected, as outlined above, from a series of seven healthy adult guinea pigs and allowed to autolyze under

TABLE 5

*Percentage of ammonia nitrogen in aseptically collected and in infected muscle used in this investigation, compared with that found by other workers, in autolysis under toluol and in infected muscle*

Per cent of total nitrogen

	FRESH	ASEPTICALLY COLLECTED OR UNDER TOLUOL		INFECTED MATERIAL	
		24 hours	48 hours	24 hours	48 hours
Aseptically collected:					
Experiment average . . . . .	0.28	0.49	0.65	1.6	7.5
Toluol:					
Reed's average . . . . .	0.38	1.05	1.93	1.09	2.42
Sanderson's average . . . . .	0.19	0.26	0.34	0.38	8.00

aseptic conditions. In this series, the non-coagulable nitrogen increased about 6 per cent (measured as per cent of total nitrogen) in 24 hours, as shown in table 6. During the next 24 hours it rose another 5 per cent, but by 72 hours increased but 1 per cent over the amount at 48 hours. These data, with those of the following tables, are plotted in figure 4. In the graphs, averages have been taken of all material at the same stage of autolysis.

The initial amount is not as great as that found in fish muscle, but the rate of increase is very much greater (figs. 2 and 4). The higher temperature at which the kidney was incubated probably accounts for this.

TABLE 6

*Percentage of non-coagulable nitrogen in fresh guinea pig kidney and after autolysing 12, 24, 48 and 72 hours*

SERIES	PER CENT OF TOTAL NITROGEN				
	Fresh	12 hours	24 hours	48 hours	72 hours
1	7.50		13.03	17.00	
2	9.59		15.94	19.35	
3	6.63		15.57		
4	5.81		13.18	22.50	
5	5.78		9.77	12.44	
6	6.75		9.28	12.90	
23	5.68	8.89	13.78		22.52
24	4.93	7.48	10.45		17.60
25	5.02	6.33	12.55		18.70
26	4.35	6.05			14.76
27	3.73	7.98	8.33		
29	5.10	8.25	10.93		18.58
30	4.62	7.33			18.71
Average...	5.81	7.47	12.07	16.84	18.48

TABLE 7

*Percentage of ammonia nitrogen in fresh guinea pig kidney and after autolysing 12, 24, 48 and 72 hours*

SERIES	PER CENT OF TOTAL NITROGEN				
	Fresh	12 hours	24 hours	48 hours	72 hours
1			0.63		
2			0.48	0.84	
3	0.11		0.55		
4	0.11		0.49		
5	0.19		0.57	0.66	
6	0.16		0.75		
23	0.13	0.41	0.61		0.85
24	0.11	0.35	0.48		0.77
25	0.12	0.25			0.70
26	0.11	0.23	0.41		0.57
27	0.11	0.33	0.44		0.66
29	0.26	0.54	0.68		0.82
30	0.24	0.48	0.63		0.81
Average....	0.15	0.37	0.56	0.75	0.75

### 9. Determination of ammonia nitrogen in sterile kidney

The increase in ammonia nitrogen in the above series is very slight, the amount at 72 hours not making up 1 per cent of the total nitrogen. This amount is actually five times that found in the fresh material, but the initial quantity is so small that this increase is not apparent (fig. 5). The greater amount of this ammonia nitrogen is produced during the first 48 hours.

### 10. Determination of non-coagulable nitrogen in kidney infected with *Proteus vulgaris* when fresh and after autolyzing 24 and 48 hours

Samples of macerated kidney, prepared as in the previous experiments, were infected by introducing a few drops of a suspension of a 24 hour culture of *Proteus vulgaris*. In table 8 are included the results obtained when organisms were introduced into fresh kidney and after autolyzing aseptically for 24 to 48 hours. These results are plotted in figure 4.

Organisms introduced into fresh kidney produce a marked change, with an increase in non-coagulable nitrogen of 10 per cent in the first 24 hours. The amount of non-protein nitrogen produced during the next 24 hours is about the same but at 72 hours it is but 3 per cent more than at 48 hours. The primary cleavage seems to be reaching an equilibrium. The production of ammonia, which we might term the secondary cleavage, is still progressing (fig. 5), and no doubt depends to a great extent on this first reaction. About 10 per cent more of the total nitrogen appears as non-protein nitrogen at 72 hours than is produced in the same length of time by the autolytic enzymes alone.

If the tissues autolyze 24 hours before *Proteus* is introduced, there is no immediate reaction on the proteins. On the contrary, in the next 24 hours the amount of non-coagulable nitrogen drops about 1 per cent below the amount produced during autolysis alone. During the next 24 hours, however, there is an increase of about 10 per cent.

When organisms are not introduced until the tissue has autolyzed 48 hours, there is a slight increase in non-coagulable nitrogen during the next 24 hours.



It seems then that, if the simpler products are available, the organisms utilize them almost as fast as they are produced. But by 24 hours either their numbers have increased so greatly,

TABLE 8

*Percentage of non-coagulable nitrogen in guinea pig kidney infected with Prot. vulgaris when fresh and after autolysing 24 and 48 hours*

SERIES	PER CENT OF TOTAL NITROGEN					REMARKS
	Fresh	12 hours	24 hours	48 hours	72 hours	
9	6.41		17.47	28.55		<i>Prot. vulgaris</i> introduced into fresh tissue
10	8.03		17.63	31.04		
17	5.54		18.37	27.20		
18	6.79		15.40	24.54		
49			18.84	31.03	32.60	
50			15.62	27.98	31.18	
52			14.52	26.61		
55				23.31	25.54	
56				20.34	26.83	
Average..	6.69		16.83	26.73	29.04	
11	4.70			14.47		<i>Prot. vulgaris</i> introduced at 24 hours
12	7.20		11.80	14.52		
13	6.52		15.67	24.52		
15	7.41		17.23	17.88		
16	5.09		12.29			
35	5.23		10.75	14.07	24.07	
36			12.73	15.21	26.75	
Average..	6.02		13.41	16.78	25.41	
39	6.24			18.78	16.50	<i>Prot. vulgaris</i> introduced at 48 hours
40	7.77				19.15	
41	5.39			16.19	18.48	
47		8.68	11.59	18.19	20.68	
48		8.68	11.60	18.29	18.21	
Average..	6.46	8.68	11.59	17.88	18.60	

or enough enzymes have been elaborated, so that a very rapid breakdown of the proteins results. Organisms in fresh tissues, however, act on the proteins almost immediately and produce very decided changes.

11. *Determination of ammonia nitrogen in kidney infected with Proteus vulgaris when fresh and after autolyzing 24 and 48 hours.*

Comparing the amounts of ammonia produced when organisms are introduced into fresh kidney with the initial amount present,

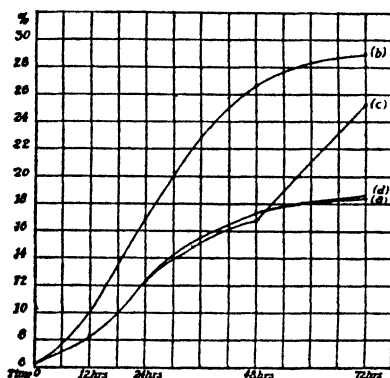


FIG. 4

FIG. 4. INCREASE IN NON-COAGULABLE NITROGEN IN GUINEA PIG KIDNEY  
 a. Sterile kidney. b. *Prot. vulgaris* introduced into fresh kidney. c. *Prot. vulgaris* introduced after twenty-four hours autolysis. d. *Prot. vulgaris* introduced after forty-eight hours autolysis.

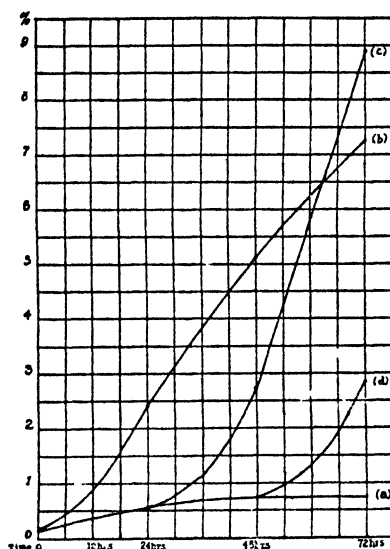


FIG. 5

FIG. 5. INCREASE IN AMMONIA NITROGEN IN GUINEA PIG KIDNEY  
 a. Sterile kidney. b. *Prot. vulgaris* introduced into fresh kidney. c. *Prot. vulgaris* introduced after twenty-four hours autolysis. d. *Prot. vulgaris* introduced after forty-eight hours autolysis.

the increases are enormous (table 9). In the first 24 hours it increased from 0.2 to 2.4 per cent, in the next 24 hours to 5.1 per cent and during the next 24 hours to 7.25 per cent.

The increase is even greater when organisms are introduced into kidney that has autolyzed for 24 hours. There is an increase of 2 per cent in the first 24 hours and of 6 per cent in the next 24 hours.

There is a similar increase in the first 24 hours when the organisms are introduced after 48 hours of autolysis.

This decided increase in ammonia when organisms are introduced into autolyzing tissue, and the slight change in non-coagulable nitrogen during the first 24 hours, show that the growth of

TABLE 9

*Percentage of ammonia nitrogen in guinea pig kidney infected with Prot. vulgaris when fresh and after autolysing 24 and 48 hours*

SERIES	PER CENT OF TOTAL NITROGEN				REMARKS
	Fresh	24 hours	48 hours	72 hours	
9	0.24	2.61			<i>Prot. vulgaris</i> introduced into fresh tissue
10	0.23				
17	0.22	2.76			
18	0.21	2.61	7.43		
49		2.69	6.35	8.64	
50		2.23	5.69	7.52	
52		1.63	4.07	6.54	
55			3.79	6.37	
56			3.49		
Average...	0.22	2.42	5.13	7.27	
11	0.15	0.78	1.38		<i>Prot. vulgaris</i> introduced at 24 hours
12	0.24	0.64			
13	0.18		3.05		
15	0.23	0.91	3.38		
16	0.18	0.77	3.46		
35	0.18	0.43	2.46	8.53	
36	0.19	0.46	2.66	9.28	
Average	0.19	0.66	2.73	8.90	
41				3.89	<i>Prot. vulgaris</i> introduced at 48 hours
47			0.72	1.74	
48			0.72	2.88	
Average...			0.72	2.83	

the organisms during this time is at the expense of the non-protein nitrogen.

### *12. Growth of Proteus vulgaris in fresh and autolyzing kidney*

In order to obtain some idea of the rates of growth of the organisms in the tissue at varying stages of decomposition,

Breed counts were done at the same time as the chemical analyses were made. Averages of these are plotted in figure 6.

The rate of growth of *Prot. vulgaris* does not seem to be influenced by the length of time autolysis has gone on before inoculation. In fresh tissue, the organisms multiply while splitting and

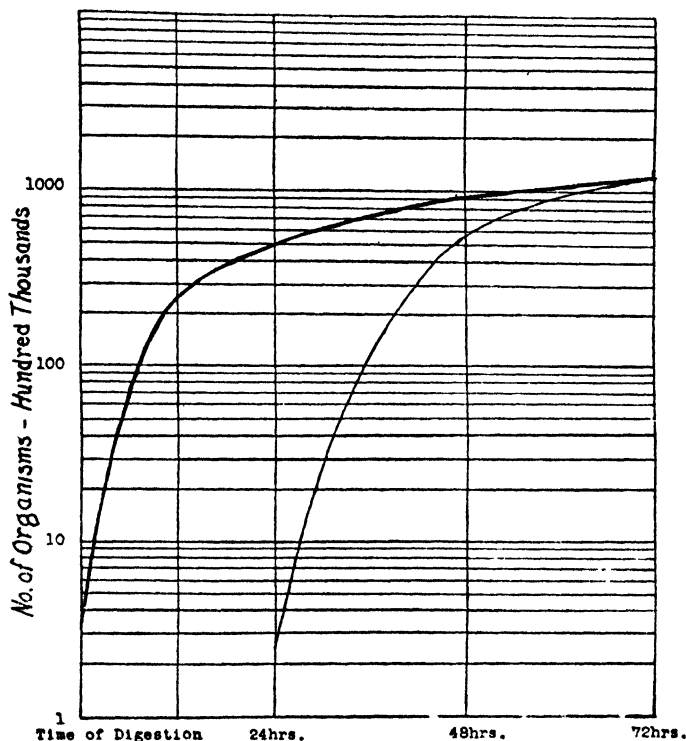


FIG. 6

Heavy line: Rate of growth of *Prot. vulgaris* introduced into fresh kidney suspension. Light line: Rate of growth of *Prot. vulgaris* introduced into kidney suspension that had autolysed twenty-four hours.

digesting the proteins, thus producing a profound and rapid change. In tissue that has autolyzed for a time, they multiply at the expense of the simpler products for 24 hours. Their numbers during this time have increased to such an extent that the proteins are then attacked much more vigorously.

## CONCLUSIONS

1. It has been shown that organisms, pure cultures or mixed infections, introduced into fresh tissue cause a rapid breaking down of the proteins and an enormous increase in ammonia.

2. *Prot. vulgaris* introduced into sterile kidney after 24 hours autolysis, first utilizes the simple products of autolysis causing a secondary cleavage. It then produces a very rapid increase in both non-protein and ammonia nitrogen. Under these conditions, the organisms produce almost as great a change in 48 hours as they do in 72 hours in fresh tissue.

3. The degree of autolysis preceding the introduction of the organisms does not affect the growth rate of *Prot. vulgaris*, but it does make a decided difference in the chemical changes produced.

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# ANAEROBIC BACTERIA IN DAIRY PRODUCTS

## I. NUMBERS OF SPORES OF ANAEROBIC BACTERIA IN MILK AND CREAM

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### INTRODUCTION

Organisms belonging to the genus *Clostridium* have frequently been found in dairy products and have been considered to be causally related to certain of the changes taking place in these materials. Their growth is undoubtedly influenced by the other organisms present. Presumably it is favored by the using up of oxygen and, on the other hand, it may be inhibited by some of the products, such as acids, that are formed by certain species of bacteria. The presence of anaerobes in a dairy product is no assurance that they will develop but it would appear that if large numbers were involved the chances for their growth would be better than if only small numbers were included.

The investigation herein reported was undertaken with the idea of securing information as to the numbers of spores of anaerobes that could be found in milk and cream produced under Iowa conditions. The work was limited to the spores because of the difficulties in including the vegetative cells. Moreover, it would seem that, because of the sources from which they come, most of the anaerobes gaining entrance to milk or cream would be in the spore stage.

### HISTORICAL

The investigations of Rodella (1904) indicated that anaerobes are present in milk to the number of from one to three per 0.1 cc. Wolff (1905) usually found anaerobes in 1 cc. portions of milk

but only once in a 0.1 cc. sample. Barthel (1910) investigated milk for the presence of anaerobes and concluded that they occurred in small numbers in commercial milk. He reported that they often could not be found even in 15 to 20 cc. portions.

A method of examining milk for spores of *Bacillus enteritidis-sporogenes* was described by Savage (1912); he concluded that it was of value in determining the purity of milk samples.

Weinzirl and Veldee (1915) examined 5, 10 and 15 cc. quantities of milk for the spores of anaerobic bacteria. With market milk the following results were secured: 90 samples were examined using 5 cc. portions and 28 per cent were positive; 112 samples were examined using 10 cc. portions and 37.5 per cent were positive; 34 samples were examined using 15 cc. portions and 50 per cent were positive. It is evident from these data that the numbers of spores of anaerobic bacteria in the milk were small. The data secured on pasteurized milk are as follows: 110 samples were examined using 5 cc. portions and 18 per cent were positive; 110 samples were examined using 10 cc. portions and 57 per cent were positive.

Ayers and Clemmer (1921) carried out investigations on the anaerobic spore test. From the results they secured it is evident that the numbers of spores were never very large.

Rawlinson (1926) examined 35 samples of London ice cream during July and August and found *Clostridium welchii* present in numbers ranging from 1 to 100,000 per cubic centimeter.

#### METHODS

The procedure used to estimate the numbers of spores present in the samples examined was an adaptation of widely used methods and involved the inoculation of different volumes of the materials to be examined into tubes of sterile litmus skim milk, after which heat was used to kill the vegetative cells; the tubes were then incubated at 37°C. and observations made for the growth of anaerobic bacteria.

Two different sized tubes were used in which to inoculate the samples. Those used for quantities ranging from 5 to 100 cc. measured 3.8 by 25 cm. and had a capacity of 200 cc., while those

for volumes of 1 cc. or less measured 1.5 by 12 cm. and had a capacity of 20 cc. Litmus skim milk was added to each of the larger tubes in such an amount that the volume after the addition of the sample would be about 100 cc., except in the case of tubes that were to receive 100 cc. of sample when 50 cc. of litmus skim milk were used. Each of the small tubes received 10 cc. of the litmus skim milk. The milk was sterilized in the tubes, cooled by holding in cold water, inoculated and then heated to 80°C. for fifteen minutes after which it was quickly cooled to 37°C. and incubated at this temperature. No seals were used on the large tubes but the material in the small ones was flooded with a sterile mixture of one part paraffin and two parts vaseline.

The volumes used for the examination of a sample, the number of tests with each volume and the amount of sterile litmus milk employed with each volume are shown in the following tabulation:

VOLUMES OF A SAMPLE USED FOR EXAMINATION	NUMBER OF TUBES	AMOUNT OF STERILE LITMUS MILK USED
cc.		cc.
100 0	2	50
50 0	2	50
25.0	2	75
10.0	2	90
5.0	2	95
1.0	5	10
0.1	5	10

Observations for evidence of the growth of anaerobes were made daily for five days. Such growth was first judged on the basis of gas production, coagulation and odor, but all positive findings were checked by the actual isolation of an anaerobe from one or more tubes of each set. From the tubes in which anaerobes developed conclusions were drawn as to the number of spores in the sample.

#### RESULTS OBTAINED

The data secured on the numbers of spores of anaerobic bacteria in pasteurized and raw milk from the market milk laboratory of the Iowa State College are given in table 1. None of the 15



TABLE 1  
*Numbers of spores of anaerobic bacteria in milk*

NUMBERS OF SPORES	NUMBERS OF SAMPLES
Pasteurized milk:	
1 spore in 10 cc.....	1
1 spore in 5 cc.....	3
2 spores in 5 cc.....	3
3 spores in 5 cc.....	2
4 spores in 1 cc. ....	2
Raw milk:	
4 spores in 5 cc.....	4

TABLE 2  
*Numbers of spores of anaerobic bacteria in cream*

NUMBERS OF SPORES	NUMBERS OF SAMPLES
Pasteurized cream:	
1 spore in 25 cc.....	2
1 spore in 10 cc.....	1
1 spore in 5 cc. ....	1
3 spores in 5 cc. ....	1
Raw cream:	
1 spore in 50 cc.....	2
1 spore in 25 cc. ....	2
1 spore in 10 cc....	3
1 spore in 5 cc. ....	1
2 spores in 5 cc.....	1
3 spores in 5 cc.....	4
4 spores in 5 cc....	3
2 spores in 1 cc....	1
4 spores in 1 cc.....	3
6 spores in 1 cc.....	1
10 spores in 1 cc.....	1

TABLE 3  
*Numbers of spores of anaerobic bacteria in sour skim milk*

NUMBERS OF SPORES	NUMBERS OF SAMPLES
2 spores in 1 cc.....	1
10 spores in 1 cc. ....	1

samples, 11 pasteurized and 4 raw, contained the spores in large numbers; the maximum number was 4 spores in 1 cc., while the minimum was 1 spore in 10 cc.

Table 2 gives the results obtained on 27 samples of sweet cream, 5 pasteurized and 22 raw, that came from either the market milk or butter laboratory of the Iowa State College. As with the milk, the numbers of the spores present in the cream were comparatively small. The maximum was 10 spores in 1 cc. and the minimum was 1 spore in 50 cc.

Two samples of condensed sour skim milk from an Iowa condensery were also examined for the numbers of spores of anaerobes contained. This product is regularly put up in barrels. No attempt is made to sterilize it, the high acidity being relied on to inhibit bacterial action. From the results obtained, which are given in table 3, it is evident that in this material also the spores were present in comparatively small numbers.

The tubes in which anaerobes developed ordinarily showed extensive changes due to these forms. Gas production and characteristic odors were conspicuous and often definitely preceded coagulation. The types of changes in the tubes varied considerably and were undoubtedly influenced by such factors as whether one or more species were present and the combinations of organisms in the mixed cultures. In some instances aerobic spore formers developed and played a part in the changes that took place. Experience made it possible to recognize such organisms by organoleptic tests, although microscopic examinations and agar shake cultures were very helpful in drawing definite conclusions.

#### DISCUSSION OF RESULTS

Spores of anaerobes were present in each of the 44 samples studied, 15 of milk, 27 of cream and 2 of condensed sour skim milk, but the numbers were always comparatively small. The maximum was 10 spores in 1 cc. and the minimum was 1 spore in 50 cc. No definite difference between the pasteurized and the raw products was evident; the 11 samples of pasteurized milk showed both higher and lower numbers of spores than the 4 samples of raw milk, while the 22 samples of raw cream showed both higher and lower numbers than the 5 samples of pasteurized cream. This indicates that, as would be expected from the

general resistance of bacterial spores, pasteurization has no effect on the spores of anaerobes.

The rapid development of anaerobes in milk in which conditions have been made favorable for these organisms suggests that the restraining action of various factors is important in preventing their growth under practical conditions. The objectionable fermentations produced by some of the anaerobes are evidence that the rapid development of acid commonly occurring in milk and cream on holding is to be preferred to some of the changes that can take place in these products.

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# ANAEROBIC BACTERIA IN DAIRY PRODUCTS

## II. RELATIONSHIP OF ANAEROBIC BACTERIA TO CERTAIN ABNORMAL FERMENTATIONS

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### INTRODUCTION

Abnormal changes occurring in dairy products under practical conditions are occasionally found to be due to anaerobic bacteria. Because of the difficulties involved in isolating and studying organisms of this type, it seems probable that they have also been of importance in fermentations in which they have been missed, especially when aerobic or facultative organisms capable of producing the same general change were also present. A number of objectionable fermentations in dairy products due to anaerobes have been encountered at the Iowa Agricultural Experiment Station; some of these are reported herein.

### HISTORICAL

Burri (1923), in discussing the relationship of silage to contamination of cheese, reported that motile butyric acid bacteria and *Clostridium putrificum* were responsible for abnormal conditions in Emmental cheese. He found the spores of *C. putrificum* regularly in Emmental cheese, but in small numbers. Burri concluded that the stinker type of cheese is caused by a combination of *C. putrificum* and butyric acid bacteria and that the numerous defective products found in American cheese factories as the result of the action of butyric acid bacteria and other spore forming organisms may be due to the use of unsuitable silage on certain farms.

Matheson (1923) isolated a spore-forming anaerobe from

typical niszler cheese and found that it could reproduce the condition in experimental cheese.

An anaerobe similar to *Clostridium butyricum* was secured from a pimento cheese of the Neufchatel type by Warren (1926). The cheese became bitter and sharply acid.

Albus (1927) isolated a spore forming anaerobe from gassy Swiss cheese containing pimentos. He concluded that the organism developed because of the carbohydrate supplied by the pimento.

Later, Albus (1928) obtained a non-pathogenic strain of *Clostridium welchii* from a Swiss cheese which had developed an abnormal gassy fermentation. The organism reproduced the condition when experimental cheese was made from milk inoculated with it.

#### EXAMPLES OF ABNORMAL CONDITIONS DUE TO ANAEROBES

##### 1. *An abnormal fermentation in acidophilus milk prepared in flasks*

A method at one time used in preparing acidophilus milk was to sterilize skim milk in 2-liter Erlenmeyer flasks and, after inoculation, incubate at 37°C. Occasionally a flask of milk treated in this manner would become very gassy; if the milk had not coagulated it would show considerable foam which was greatly increased by shaking, while, if it had coagulated, the curd would be full of gas holes that conspicuously increased the volume of the material.

Microscopic examinations of the abnormal fermented material revealed the presence of considerable numbers of short, thick, Gram-positive rods among the *Lactobacillus acidophilus* cells. The numbers of these rods and the absence of other contaminating types indicated that they were responsible for the abnormal condition, while the general morphology suggested that they were anaerobes. Isolations were made by means of agar shake cultures and only the one contaminant was found. It possessed morphological and cultural characters which classed it as *C. welchii*.

2. *An abnormal fermentation in acidophilus milk prepared in a tank*

Acidophilus milk was also prepared by heating milk in a 20-gallon, glass-lined, insulated tank under about 6 pounds steam pressure for one hour, after which it was cooled to 37°C. and inoculated through a small opening in the top of the tank. Usually, twenty-four hours were required to develop the desired acidity.

On one occasion, when the tank was opened for examination about twenty hours after inoculation, a floating curd full of gas holes was found. A microscopic examination of this material showed that along with the *L. acidophilus* cells there were a number of short, thick, gram positive rods. When inoculations were made from the fermented milk into brain medium containing 0.1 per cent glucose there was pronounced gas formation and microscopic observations showed that the thick Gram-positive rods had become very numerous.

Although detailed studies of the contaminating organism were not made, the general characters indicated that it was *C. welchii*.

3. *Coagulation and gas development in pasteurized milk*

During a study of the changes occurring in pasteurized milk at different temperatures, two samples held at room temperature coagulated and became very gassy. Each of these was examined microscopically and numerous Gram-variable rods were found.

Shake cultures of the samples were made in glucose agar and, after incubation for twenty-four hours at 37°C., colonies were visible. Some of these were picked into freshly boiled and cooled litmus milk and the tubes sealed with a mixture of paraffin and vaseline. Upon incubation at 37°C., coagulation and gas production occurred.

The microscopic and cultural characters of the organisms isolated indicated that they were of one type and that they belonged to the group of anaerobic organisms of which *C. butyricum* is a member. Until more work has been done on this group of organisms, it will be impossible to identify the isolated cultures definitely.

#### 4. *Gas production in pasteurized milk*

During later work on the changes taking place in pasteurized milk held at room temperature, a considerable number of samples showed gas development in from three to four days. Usually coagulation did not occur until several days after the beginning of the gas production. The samples of milk showing the gassy condition generally had a rather pronounced rancid odor.

Microscopic examination of material from the samples showed the presence of streptococci, Gram-negative rods and Gram-positive rods. The Gram-positive rods were of various sizes and appeared to include different species. Some of them were large and thick and had terminal to subterminal oval spores; what was believed to be this type was isolated by means of liver infusion glucose agar shake cultures.

In pure culture, the organism appeared as a fairly long, thick, motile rod that stained Gram-variable with most of the rods negative, even in twenty-four-hour cultures. Spores were formed in twenty-four hours in 0.1 per cent glucose brain medium; they were oval and occurred in terminal to subterminal positions. The rods carrying spores were swollen. Litmus milk under a seal was reduced, gas was formed and a soft curd furrowed by escaping gas developed. In 0.1 per cent glucose brain medium, gas was produced but there was no blackening or softening. The organism undoubtedly belongs to the group of butyric acid organisms.

#### 5. *Rancid odor in pasteurized cream*

A number of samples of pasteurized table cream, which had been held over-night in a comparatively warm cellar because ice was not available, developed a rancid odor. Following this, the cream coagulated and, with a curd present, gas production was evident.

In each instance where this rancid condition was noted, an anaerobe that was believed to be the cause was isolated. The cultures were of two types. One type possessed characters identifying it as *C. welchii*, while the other was the same as the organism described under example 4.

## SUMMARY

Instances of abnormal conditions in dairy products caused by the growth of anaerobic bacteria are reported. The organisms involved were *C. welchii* and types belonging to the group of butyric acid organisms.

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# A SIMPLE METHOD OF GROWING ANAEROBES IN PETRI DISH CULTURES

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The literature dealing with methods of growing anaerobes has become very extensive. An investigation of the methods used shows that the removal of oxygen has been brought about by one or more of the following ways: (1) Evacuation, (2) sweeping away of the air by means of a so-called inert gas, (3) removal of air by heat before inoculation and subsequent hinderance or prevention of oxygen diffusion, (4) absorption of oxygen by chemicals or by aerobic microorganisms. For a review and description of the various methods the reader is referred to the papers by Roux (1887), Novy (1893), Hunziker (1902), Schattenfroh (1923), Zeissler (1923), Hall (1929), and others.

It is worth noticing that none of the methods recommended in the literature for petri dish cultures is practical and efficient enough for frequent and extensive work and some of them are open to serious objections. It is the hope of the authors that the method described in this paper will answer the need of various laboratories because of its simplicity and efficiency and because it requires no special equipments or materials other than those usually present in any bacteriological or chemical laboratory; namely, a vacuum desiccator and a suction pump.

The petri dishes are poured as for aerobic cultures, except that a small amount of agar, not exceeding 8 cc. is used. The plates are allowed to harden and are then inverted in a vacuum desiccator over a dish containing water at about 45°C. The water may be placed directly at the bottom of the desiccator. It is desirable that the desiccator walls and cover be warmed gently to about 45°C. The desiccator is then covered, connected with

a suction pump and evacuated slowly until the water boils, and its vapor sweeps away the last traces of air. Just before boiling ceases, the desiccator is closed and incubated at the desired temperature for the desired length of time. The rate of evacuation should be such that thirty minutes or more is required for completion.

The method is, therefore, a combination of evacuation and the use of water vapor to sweep away the air which has, as compared with the use of the so-called inert gases, the advantages that it is the same substance as that in which the bacteria are living and that no additional expense or special equipments are required. It offers for the growth of bacteria an ideal atmosphere saturated with water vapor which, at the same time, prevents the drying up of the agar, no matter how long the period of incubation may be.

The object of using warm water is to promote its boiling under the vacuum afforded by the suction pump, and the walls and covers of the desiccators are warmed to prevent the condensation of the vapors on those surfaces which would cut down the efficiency with which air is removed. Slow evacuation is necessary to allow the diffusion of dissolved gases and to prevent the tearing of agar to pieces by liberated gas bubbles. Finally, the agar layer must be thin to make easy the diffusion of gases and, especially, to prevent falling of the agar which, in absence of an adequate pressure below, otherwise collapses under its own weight.

The efficiency of the method is very high. The authors were able, for instance, to grow without any difficulty, strict anaerobes like *Cl. welchii*, *Cl. sporogenes* and *Cl. botulinum*, and to keep reduced methylene blue in the desiccator for ten days without the slightest return of color. Moreover, several experiments have been run to determine the pressure inside of the desiccator and the temperature of the water after evacuation. It was found in one of these experiments, for instance, that the pressure was 35 mm. of mercury and the temperature of the water 31.8°C. The vapor tension of water at that temperature is given as 34.973 mm. showing that the air pressure inside of the desiccator has been reduced to a limit that cannot be measured with the means at hand.

It is the hope of the authors that this method will fill a need that, so far, has not been filled by any of the methods we find in anaerobic literature.

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# A METHOD FOR PREPARING PHOTOGRAPHS OF PETRI DISH CULTURES BY DIRECT CONTACT PRINTING ON PHOTOGRAPHIC PAPER

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Recently we discovered that photographs of Petri dish cultures prepared by direct contact printing on photographic developing paper are as satisfactory for most purposes as pictures obtained by the use of a camera and the usual method of photographic technique. The Petri dishes are used in the same manner as negatives and the resulting prints are positives, in natural size, the colonies appearing as white objects on a black background. The details of such forms as *B. mycoides* are reproduced with striking clearness (fig. 1). Because of the simplicity of the process, the excellence of the product and the many uses to which it may be put by investigators and teachers, we have thought it desirable to describe this method in some detail.

The entire process is carried out in a dark room, using a ceiling lamp as a source of light for printing. At a distance of 2.5 to 3 meters the rays which reach the object, from a concentrated source of light, are very nearly parallel and cast dark, sharp shadows of the colonies on the white paper. We have used an ordinary clear glass 150 watt Mazda lamp at 3 meters distance from the object. A special opaque lamp shade is probably of no great advantage, when the ceiling and walls are blackened, but we have used a cylindrical shade painted black on the inside and provided with a 60 mm. opening below in order to prevent reflections from the walls of the room. The light must be centered directly above the exposing table so that the sides of the dish cast no shadows.

All preliminary operations preceding the exposure may be

carried out by the use of conveniently placed ruby or amber lights. The Petri dish, with cover removed, is placed bottom side down on the sensitive side of the paper and pressed down. A piece of cloth under the paper facilitates close contact which is necessary. A special plate holder seems to be unnecessary.

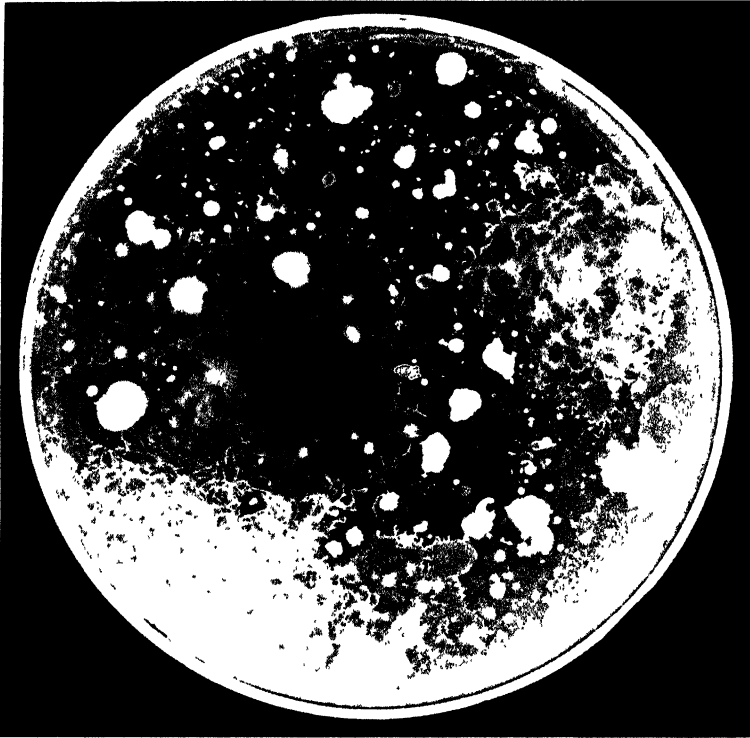


FIG 1 PHOTOGRAPH OF A POURED DILUTION PLATE BY DIRECT CONTACT PRINTING ON PAPER

When plate and paper are properly adjusted the ceiling light is turned on for the desired exposure. The period of exposure depends on several factors and needs to be determined for each culture. The color and transparency of the agar, its depth in the dish, the kind of paper, the developer used, and the temperature of development all influence the time required. With an agar which is almost colorless, and with vigorous Novagas paper, the

exposure may be as short as 10 seconds but with No. 4 Azo the same subject may require 150 seconds. Agar or gelatin which has more of the amber color, due to beef extract or beef infusion, requires a longer exposure. We adjust the time of exposure so that development of the print is completed in 35 to 60 seconds.

The kind of paper used is the most important single detail. Bromide papers are too fast, requiring such short exposures (usually about  $\frac{1}{2}$  second) that there is difficulty in properly timing the exposure, and they also lack contrast. If Azo paper is used it should be No. 4 or No. 5; Nos. 1 and 2, in our experiments, always gave unsatisfactory results; No. 3 may be useful for an unusual subject. Excellent results were also obtained by using Gevaert's Novagas vigorous paper. This paper has the advantage of requiring a shorter period of exposure. Extra vigorous Novagas was not tested but is probably still more desirable for very transparent subjects. Both Novagas and Azo papers give satisfactory detail and contrast.

The kind of developer appears to be of considerable importance. The standard Eastman "MQ" Elon Hydrochinon developer is less satisfactory than the Eastman Special, which may also be obtained in tubes. We have tested several formulae and found the following very satisfactory.

*Formula D72. Stock solution*

Water.....	500 cc.
Elon (Photol or Metol).....	3 grams
Sodium sulphite.....	45 grams
Hydrochinon.....	12 grams
Sodium carbonate.....	65 grams
Potassium bromide.....	1.8 grams
Water to make.....	1 liter

For use take 1 part stock solution to 1 part water.

*Formula D73. Stock solution*

Water.....	500 cc.
Elon (Photol or Metol).....	2.7 grams
Sodium sulphite.....	40 grams
Hydrochinon.....	10.6 grams
Sodium carbonate.....	75 grams
Potassium bromide.....	.8 grams
Water to make.....	1 liter



For use take 1 part stock solution to 2 parts water. Formula D72 gives warmer tones and works faster than D73, but either of these formulae will give good results. D73 diluted 1:2 seems to

TABLE 1

*Exposure and development time table for an average subject with various brands of paper and kinds of developer*

The source of light was a clear glass 150 watt Mazda Lamp at a distance of 3 meters. Time is given in seconds.

	AZO NO. 5 RECOM- MENDED		AZO NO. 4 RECOM- MENDED		AZO NO. 3 INFERIOR FOR OUR SUBJECTS		GEVAERT'S NOVAGAS (VIGOROUS) RECOM- MENDED		BROMIDE (NOT RECOM- MENDED)	
	Exposure	Develop- ment	Exposure	Develop- ment	Exposure	Develop- ment	Exposure	Develop- ment	Exposure	Develop- ment
Eastman Special 75- 76°F. recommended	90	40	150	50	30	40	30 15 10	25 45 60	1?	180
Eastman Formula D72	120	40	150	50						
Diluted 1:1 77°F. recommended	105	50								
	90	65								
Eastman "MQ" Elon- Hydro. not recom- mended	210	25	300	35		25				
	180	40	240	45	60	35				
	150	70	180	90	30	50				
					AZO NO. 2 VERY INFE- RIOR NOT RE- COMMENDED					
Eastman Formula D73	240	30	360	25	180	30				
full strength stock	180	45	300	45	120	45				
solution 77°F.	120	60	240	55	90	90				
Eastman Formula D73	180	35								
diluted 1:2 parts	150	45								
water 77°F.	120	60								
	90	90								

work faster than the stock solution. For a convenient quickly prepared developer we recommend Eastman's Special. Table 1 showing the periods of exposure and development required to give

satisfactory results, using the several brands and grades of paper and kinds of developer which we have tested, suffices to indicate the time relations to be expected for an average subject.

The Petri dishes for cultures which are to be photographed should be selected with care. They should have flat bottoms, free from scratches and other imperfections. Care should be exercised to see that the layer of agar is not too deep and that its thickness is uniform throughout.

Sensitized plates or films may be used in the same manner as paper if desired. Since these have a bromide emulsion they must be handled only in ruby light, and their time of exposure is very short, only a fraction of a second, with a less powerful ceiling lamp. A slow emulsion is preferable. We have used Eastman's Process films, developed with a contrast developer (D11), with very satisfactory results. The time of development may be varied in order to give different degrees of contrast. These films give positives in the same sense as the contact prints described above, and the prints made from them are reversed, black colonies against a white background. If white colonies against a black background are desired from such films, it is necessary to print the film image in a printing frame on another film which is developed and used as a negative for the final prints. When films are used in making the first impression, the differences due to the variations in the thickness of the agar in various parts of the Petri dish are less pronounced. Otherwise the use of films offers no great advantages. The accompanying illustration (fig. 1) of a soil dilution plate was prepared by direct contact printing on Azo paper No. 4.

A survey of the literature revealed the fact that others have employed methods similar to the one developed in our work but differing from it in certain important details. De Giaksa (1888) appears to have been the first worker to describe a direct printing method for this purpose. At this early date the original Koch plates were still in use. He placed such a plate on a piece of sensitized printing out paper, exposed to sunlight, and then treated the print according to the toning and fixing methods in use at that time. The illustration which accompanies his description of the

process is remarkable for the differentiation and contrast which it shows. De Giaksa pointed out the advantages of such a method and the principles involved in the process, but the method does not appear to have come into general use, if one may judge by the published work which followed.

Lindner (1914) described a photographic method based on a process of shadow printing, using various biological objects placed directly on photographic paper. He employed an arc lamp and mirror, describing the parallel rays as an essential condition of his process. The Petri dish cultures which he has illustrated show the usual white colonies against a dark background. In a more extensive treatise (1920) this author enlarges on the general subject of photography without the use of a camera, but this book has not found its way into many American libraries.

Broadhurst (1917) described a method of printing from agar plates directly on blue-print paper. She called attention to the simplicity of the process and its usefulness in the preparation of demonstration material for teachers of bacteriology. Since blue-prints are not suitable for reproduction, illustrations of this method are lacking. It is possible that others may have described a similar procedure but if so we have not found reference to it in a search of the literature.

The method which we have employed appears to be more simple than any which we have seen described. We have used it for the reproduction of many types of Petri dish cultures including exposure plates; water, milk and soil analyses; effect of light, disinfectants and dyes; bacterial antagonism; liquefaction of gelatin and effect of bacteria on gelatin in Frazier's medium. The method is admirably suited to the needs of students who wish to preserve results obtained in class work. Students who have had no previous experience with photography have been able, by this method, to obtain excellent illustrations of plates prepared in routine laboratory experiments. We believe the method will be found very useful by teachers of bacteriology.

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# THE CELL STRUCTURE AND CELL DIVISION OF *BACILLUS SUBTILIS*

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If the cell of *Bacillus subtilis* be stained vitally with an aqueous solution of crystal violet containing 1 gram of the dye per liter, it shows a purple outer cell wall surrounding a dark violet membrane or ectoplasm which encloses a deeply staining cytoplasm but of a much lighter shade (fig. 1). If the cells are mounted in a concentrated salt solution (a 25 per cent sodium chloride solution for example) previous to staining, many will be plasmolyzed, and the cell wall is then seen clearly surrounding a whole chain with frequent septations between cells; and when two cells or two chains lie contiguous, the double thickness of the wall makes it stand out more clearly (fig. 2). The ectoplasm shows thickened areas and appendages of various sizes and shapes on its internal surface similar to those described by the author (1929) for *Proteus vulgaris* and for *Mycobacterium tuberculosis*, and is drawn in with the cytoplasm when the cell is plasmolyzed.

The cell wall has only a slight affinity for dyes and stains a clear blue with methylene blue. It has no affinity for iodine. The ectoplasm, on the other hand is, like that of other bacteria, hyperchromatic, and takes up dyes with great avidity. It is colored dark brown with iodine.

Cell division can be best followed in cells mounted in Lugol's solution which stains only the ectoplasm to any extent. The extensions of the ectoplasm which are clearly seen on opposite sides of the cell in two, three, or more places grow in and at the same time grow in thickness. They finally meet and form a band through which a very clear sharply defined colorless line of division appears. The other extensions ultimately do the

same and so on (fig. 3, *a-d*). In young cultures, the daughter cells keep on growing in size and dividing again, but in older ones, where growth has become slow, the daughter cells do not increase

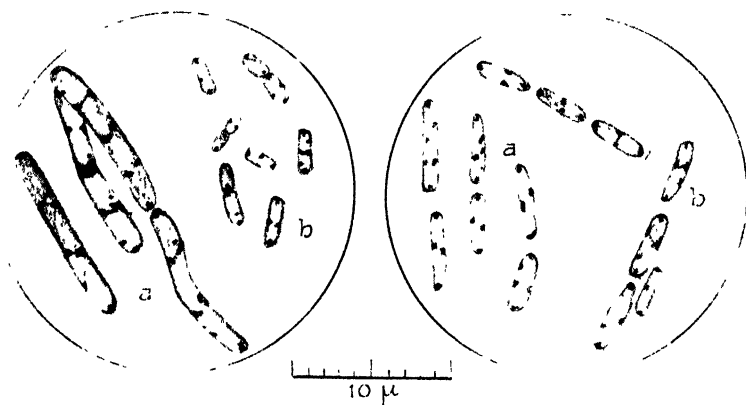


FIG. 1

FIG. 2

FIG. 1. *a*, four-hour old cells mounted in 1/1000 aqueous crystal violet solution. *b*, twenty-four hour old cells stained as in *a*

FIG. 2 *a* and *b*, six-hour old cells mounted in a drop of a 25 per cent sodium chloride solution and stained as in figure 1.

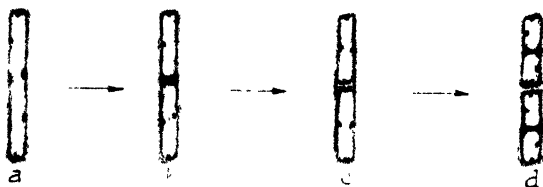


FIG. 3. *a-d*, CONSECUTIVE STAGES IN CELL DIVISION SHOWING ALSO THE MECHANISM OF REDUCTION OF CELL SIZE WITH AGE

in size fast enough and the results will be a remarkable decrease in the size of the cells.

Of the above structures, the ectoplasm is the one responsible for the Gram reaction and corresponds to Churchman's (1927a) cortex. On losing this structure, the cells become Gram-negative

as has been shown by Eisenberg (1909) and much more strikingly by Churchman (1927a, 1927b).

These investigations and unpublished results with other species show that bacteria are not easily affected by osmotic pressure. Moreover, they do not bear out Brudny's (1908) and Eisenberg's (1910) assertions that gram-positive bacteria are not plasmolyzable.

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# GROWTH OF ANAEROBES IN CRYSTAL VIOLET BILE MEDIA

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While experimenting with crystal violet bile media for use in water analysis, it was thought that some information could be obtained by testing the growth of anaerobes in media containing crystal violet and bile.

An ideal medium for the detection of pollution in water supplies would be, of course, a medium which would prevent the growth of all bacteria except members of the colon group. This, however, is far from possible. *B. welchii* and other anaerobes are responsible for many false presumptive tests in waters. This fact was first reported by Frost (1906) in 1906. Many investigators have observed this condition in recent years.

The question arose as to whether crystal violet bile broth would be effective in preventing the growth of anaerobes. Apparently this subject has not been investigated in regard to water analysis as no reference could be found in the literature. Hall and Ellefson (1918) (1919) were the first to call attention to the fact that gentian violet, when added to lactose broth, would eliminate the false presumptive test due to anaerobes. They found that one part in 20,000 was effective in eliminating all gas-forming anaerobes. Wagner and Monfort (1921) confirmed the work of Hall and Ellefson.

## EXPERIMENTAL

Lactose broth was prepared and varying amounts of crystal violet and bile were added. The strength of the lactose and peptone was 1 per cent each. The pH value of all media was

between 7.2 and 7.3. The media, in 10 cc. lots, were placed in Durham fermentation tubes. Some of the media, without the dye, was also prepared so that the effect of the bile could be studied. Dye in different concentrations was also added to the plain lactose broth.

Suspensions of the different anaerobic cultures to be tested were made in sterile distilled water. Five cubic centimeters of this dilution were added to one tube from each of the different lots of media. The tubes were allowed to incubate at 37°C., and gas readings were made in twelve, eighteen, twenty-four, and forty-eight hours. In case there was no gas production in forty-eight hours, another reading was made in seventy-two hours. In the routine methods for water analysis 10 cc. of water are added to 20 cc. of medium. These proportions were used throughout this work. All results are expressed in terms of the amount of dye present in the original medium before the sample of inoculum was added.

The cultures used were mostly obtained from Dr. Ivan C. Hall, University of Colorado Medical School, Denver, Colorado. Three cultures of *B. welchii* were obtained from F. E. Greer, Department of Health, Chicago. In all, nineteen cultures were used as follows: *B. multifementans*, two cultures; *B. tertius*, two cultures; *B. sphenoides*, two cultures; *B. welchii*, Type I, two cultures, Type II, three cultures, Type III, four cultures, and Type IV, four cultures.

The growth of the anaerobes in the different media will be discussed under three headings: bile media, crystal violet lactose media, and crystal violet bile media.

#### BILE MEDIA

In order to study the effect of bile on the growth of anaerobes each culture was tested with media ranging in bile content from 1 to 15 per cent. Table 1 represents the gas production for all cultures except those of *B. welchii*.

From table 1 may be seen that the growth in all dilutions of bile media was very good in the case of *B. multifementans* and *B. tertius*. With one culture of *B. sphenoides* no gas production was

apparent in any of the media, while the gas production with the other culture was very weak even in forty-eight hours.

TABLE 1  
*Gas production in per cent. Anaerobes in bile media*

PER CENT BILE	B. MULTIPERMENTANS						B. TERTIUS						B. SPHENOIDES							
	A			B			A			B			A				B			
	12 hours	18 hours	24 hours	12 hours	18 hours	24 hours	12 hours	18 hours	24 hours	12 hours	18 hours	24 hours	12 hours	18 hours	24 hours	48 hours	12 hours	18 hours	24 hours	48 hours
0	0	20	35	0	30	60	0	15	25	30	35	35	0	10	20	75	0	0	0	10
1	15	20	20	0	5	20	10	15	15	10	15	15	0	0	0	5	0	0	0	0
2	30	40	40	0	15	25	20	25	25	25	25	25	0	0	0	10	0	0	0	0
3	35	45	45	0	15	30	0	25	30	25	30	30	0	0	0	15	0	0	0	0
4	40	50	50	0	20	40	20	25	25	20	25	30	0	0	0	20	0	0	0	0
5	50	60	60	0	5	45	25	30	35	35	40	40	0	0	0	25	0	0	0	0
6	40	45	45	0	5	50	25	30	35	40	45	45	0	0	3	20	0	0	0	0
7	60	65	65	0	5	55	30	35	40	35	50	50	0	0	0	10	0	0	0	0
9	50	60	65	0	5	50	25	35	40	20	45	55	0	0	0	0	0	0	0	0
11	10	75	100	0	10	40	30	55	65	45	65	75	0	0	0	0	0	0	0	0
13	25	100	100	0	20	100	25	50	60	45	50	75	0	0	0	0	0	0	0	0
15	5	100	100	0	5	65	40	60	70	20	60	80	0	0	0	0	0	0	0	0

TABLE 2  
*Gas production in per cent with B. welchii, Type IV*

PER CENT BILE	A				B				C				D			
	12 hours	18 hours	24 hours	48 hours	12 hours	18 hours	24 hours	48 hours	12 hours	18 hours	24 hours	48 hours	12 hours	18 hours	24 hours	48 hours
0	0	0	0	10	0	10	10	10	0	0	0	10	0	0	10	20
1	0	0	5	25	0	0	0	0	0	0	0	0	0	0	15	20
2	0	0	35	35	0	0	0	0	0	0	0	0	0	10	50	50
3	0	0	5	65	0	5	10	10	0	0	0	0	0	50	60	60
4	0	0	25	70	0	0	5	10	0	0	0	0	0	55	70	75
5	0	0	60	70	0	0	10	10	0	0	0	0	0	70	80	80
6	0	0	60	60	0	0	5	10	0	0	0	0	0	65	70	70
7	0	0	65	70	0	5	10	10	0	0	0	0	0	55	70	70
9	0	0	70	85	0	0	10	15	0	0	0	0	0	80	100	100
11	0	0	0	100	0	35	50	70	0	0	10	10	0	100	100	100
13	0	0	0	100	0	0	0	10	0	65	75	80	0	90	100	100
15	0	0	0	90	0	0	0	0	0	5	15	15	0	50	100	100

The gas production with most of the strains of *B. welchii* was very good. One of the cultures of Type I gave an average of 65 per cent gas in twelve hours. The other gave 30 per cent gas in twelve hours. Two of the strains of Type II gave an average of over 40 per cent gas in twelve hours, while the other two strains averaged about 30 per cent gas in eighteen hours. All four strains of Type III averaged over 30 per cent gas in all the different strengths of bile in twelve hours. The gas production in the strains of Type IV varied considerably. The gas production for these is given in table 2. Table 3 gives the average gas production for the other strains of *B. welchii*.

TABLE 3  
*Average gas production in per cent. B. welchii, Types I, II, III*

PER CENT BILE	12 HOURS	18 HOURS	24 HOURS	48 HOURS
1	14	23	29	33
2	18	31	39	43
3	32	46	53	55
4	31	48	53	55
5	38	59	67	68
6	33	54	59	61
7	51	58	65	67
9	38	64	72	74
11	45	72	78	80
13	40	60	78	86
15	26	61	83	92

From tables 1, 2, and 3 we may conclude in general that no amount of bile present in lactose broth up to 15 per cent has the power to inhibit the growth of anaerobes.

#### CRYSTAL VIOLET LACTOSE MEDIA

As stated in the introduction Hall and Ellefson (1918) (1919) successfully used gentian violet in lactose broth to inhibit anaerobes.

Fourteen different samples of lactose broth, each containing a different amount of crystal violet, were made up. The dye content ranged from one part in 300,000 to one part in 1000.

Each of the anaerobes was tested in these media. The organisms showing growth in forty-eight hours are given in table 4.

It will be noted from table 4 that the only organisms producing gas in forty-eight hours were the *B. welchii* cultures belonging to Type II. One culture of *B. sphenoides* produced a small amount of gas in seventy-two hours in the three greatest dilutions of dye. Two cultures of other types of *B. welchii* produced a small amount of gas in seventy-two hours. One of these produced gas in a dye content as high as 1 to 75,000. In the other case the dye content was 1 to 150,000. It seems

TABLE 4

Gas production in per cent. Anaerobes in crystal violet lactose broth, *B. welchii*, Type II

DRY CONTENT	A				B				C			
	12 hours	18 hours	24 hours	48 hours	12 hours	18 hours	24 hours	48 hours	12 hours	18 hours	24 hours	48 hours
1:300,000	10	30	35	40	25	40	45	50	20	35	40	50
1:200,000	35	55	60	65	30	45	50	55	30	45	50	60
1:150,000	30	45	50	60	30	40	45	50	35	40	50	60
1:100,000	30	45	50	60	0	15	35	50	25	40	50	75
1:75,000	10	40	50	60	0	25	40	50	15	35	45	55
1:50,000	0	25	40	60	0	20	30	55	0	20	35	55
1:35,000	0	0	10	45	0	0	5	45	0	0	15	40
1:30,000	0	0	15	45	0	0	10	55	0	0	7	45
1:25,000	0	0	30	45	0	0	0	25	0	0	0	10
1:20,000	0	0	0	5	0	0	0	25	0	0	0	0

rather strange that *B. welchii*, Type II, was the only type which showed gas production in the crystal violet lactose broth. This fact might afford a means of differentiating this type of *B. welchii* from the other types. Further work should be conducted along this line by some one interested.

#### CRYSTAL VIOLET BILE MEDIA

The gas production with anaerobes in crystal violet bile media varied considerably. With the two cultures of *B. multifementans* a dye content of 1:1000 was required to inhibit growth in most of the concentrations of bile. With the cultures of *B. tertius* a

TABLE 5  
*Reciprocals of dye content required to prevent gas production in forty-eight hours*

ORGANISM	BILE (PER CENT)											
	1	2	3	4	5	6	7	9	11	13	15	
Multifermentans	{A.....	100,000	10,000	6,500	1,000	1,000	1,000	1,000	>1,000	>1,000	6,500	3,500
	{B.....	75,000	10,000	3,500	3,500	3,500	1,000	1,000	1,000	1,000	1,000	1,000
Tertius	{A.....	10,000	3,500	1,000	1,000	1,000	1,000	>1,000	>1,000	>1,000	>1,000	>1,000
	{B.....	10,000	6,500	1,000	1,000	1,000	1,000	>1,000	>1,000	>1,000	>1,000	>1,000
Sphenoides	{A.....	100,000	100,000	100,000	100,000	100,000	100,000	100,000	100,000	100,000	100,000	100,000
	{B.....	100,000	100,000	75,000*	75,000*	50,000*	75,000*	100,000	100,000	100,000	100,000	100,000
Welchii, Type I	{A.....	35,000	10,000	3,500	3,500	3,500	3,500	3,500	3,500	3,500	3,500	3,500
	{B.....	100,000	75,000	75,000	75,000	50,000	35,000	20,000	20,000	15,000	15,000	15,000
Welchii, Type II	{A.....	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000
	{B.....	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000
	{C.....	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000
Welchii, Type III	{A.....	3,500	3,500	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000	>1,000
	{B.....	75,000	25,000	6,500	6,500	3,500	3,500	3,500	3,500	3,500	3,500	3,500
	{C.....	50,000	10,000	3,500	3,500	3,500	6,500	3,500	3,500	3,500	3,500	3,500
	{D.....	50,000	6,500	6,500	3,500	3,500	3,500	3,500	3,500	3,500	3,500	3,500
Welchii, Type IV	{A.....	50,000	10,000	3,500	3,500	3,500	3,500	3,500	3,500	3,500	3,500	3,500
	{B.....	75,000	15,000	6,500	3,500	3,500	3,500	3,500	3,500	3,500	3,500	3,500
	{C.....	100,000	100,000	100,000	100,000	100,000	100,000	100,000	6,500	6,500	3,500	3,500
	{D.....	25,000	6,500	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000

\* Strengths to inhibit gas production in seventy-two hours. No gas in any dye dilution in forty-eight hours.

dye dilution of 1:1000 was required to inhibit growth in most of the higher concentrations of bile. Even with this dye concentration, gas was produced in eighteen hours with the media having a bile concentration of 9 per cent and more. With the cultures of *B. sphenoides* the growth was very scant. There was no gas production with one of the cultures. In case of the other culture no gas appeared until after seventy-two hours and this was confined to bile percentages of 3 to 7 per cent inclusive, dye content 1:100,000.

In analyzing the results from the study of the cultures of *B. welchii* we find considerable variance. One of the cultures of Type I required a dye content of 1:3500 to prevent growth. All the cultures of Type II gave excellent results in twelve to eighteen hours in a dye concentration of 1:1000. Three strains of Type III required a dye concentration of 1:3500 to prevent growth. In the other strain, excellent gas production was produced in forty-eight hours in a dye concentration of 1:1000. The members of Type IV varied the most. Two of the strains required a dye content of 1:3500. Another produced no gas in forty-eight hours in a dye concentration of 1:1000, and the fourth strain was inhibited by a dye content of 1:100,000 in the percentages of bile below 9 per cent.

In table 5 are summed up the results with the bile dye media. In this table are shown the reciprocals of the dye concentration required to prevent gas formation in forty-eight hours. A number of the cultures were not inhibited by a dye concentration of 1:1000, the greatest concentration tried. In such cases these are indicated by >1000, meaning that a dye concentration greater than 1:1000 was required to inhibit growth.

Table 5 seems to indicate that a very high concentration of dye is required to inhibit the growth of anaerobes. The concentration of dye would be far too great to use in media for water analysis.

#### CONCLUSIONS

1. In general, a bile content up to 15 per cent is not effective in eliminating the growth of anaerobes.



2. With most strains of *B. welchii* the gas production was much better in the higher percentages of bile.

3. A crystal violet dye content of over 1:75,000 in lactose broth will eliminate most anaerobes.

4. *B. welchii*, Type II seems to be very resistant to crystal violet in lactose broth. A dye content of more than 1:20,000 is required to inhibit growth in this type of *B. welchii*.

5. The addition of bile to crystal violet lactose broth destroys the inhibitive power of the dye as far as the growth of anaerobes is concerned.

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# A STUDY OF METHODS FOR THE ESTIMATION OF REDUCING SUGAR IN BACTERIOLOGICAL MEDIA

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## INTRODUCTION

In a projected study of the carbohydrate metabolism of bacteria, it was found necessary to determine accurately the amount of reducing sugar in the culture media. The only available study of the quantitative estimation of glucose in bacteriological media is that of Stiles, Peterson and Fred (1926) who used the Shaffer-Hartmann method (1921). They first clarified the media with lead subacetate and removed any excess of lead with disodium phosphate. Surprisingly enough, they found that media without clarification gave results comparable in the majority of cases with those obtained from the media after clarification. From these results one might conclude that the nitrogenous material present in the meat infusion broth had no modifying effect on the reduction of the Shaffer-Hartmann reagent by glucose. Since this method was rapid and apparently accurate, it promised to be of use for our purposes.

However, when we attempted to apply this method to meat infusion broth, the values obtained showed variations as great as 20 per cent and it was not possible to obtain consistent recoveries of added glucose.

Since the accuracy recorded by Stiles, Peterson and Fred could not be duplicated, it seemed necessary to investigate the applicability of various methods for the determination of reducing sugar in the media. The Shaffer-Hartmann and the Benedict colorimetric (Benedict (1926)) methods were tried with the filtrates of media treated with various protein precipitants.

## EXPERIMENTAL

Meat infusion broth was used throughout the experiments. It was made in the usual manner with one modification; instead of allowing the meat to be extracted in the cold for twelve hours, the water-soluble constituents were removed by autoclaving for two periods of ten minutes each at a pressure of 15 pounds. The medium was sterilized in 50 cc. portions in small flasks and kept in the refrigerator until needed. The final pH was 7.4, and all tabulated values were obtained on portions of the same preparation.

The Shaffer-Hartmann method was used as originally described (1921).<sup>1</sup> Duplicate determinations were always made upon each sample of filtrate; when the recovery of added glucose was to be estimated, these samples were treated at the same time and under the same conditions as the medium. In the recovery experiments the glucose was always added to the medium as part of the dilution, previous to any further treatment. Control determinations on a pure glucose solution were made at frequent intervals in order to check the method.

The method of Stiles, Peterson and Fred for the clarification of the media was carried out as follows: 5 cc. of medium were placed in a 50 cc. volumetric flask and made neutral to phenolphthalein; 1 cc. of lead subacetate was added (30 per cent solution of Horne's anhydrous lead subacetate), followed by 3 cc. of 10 per cent disodium phosphate solution, to remove any excess lead; the solution was again brought back to neutrality, diluted, and centrifuged after standing for five to ten minutes.

The amount of reducing material found in meat infusion broth, as determined by this method, is given in table 1. As stated above, each sample was sterile, determinations being made as soon as the flask of medium was opened. Columns 2 and 3 show that great variations occur between the duplicates of a sample,

<sup>1</sup> To obtain consistent results with this method it is necessary to weigh the potassium iodate on the analytical balance. The 0.005 N sodium thiosulfate was prepared every four days, as it is comparatively unstable (Somogyi, 1926). Any application of the Shaffer-Hartmann method must be carefully controlled; the copper reagent is subject to change—in all probability a change in pH due to loss of CO<sub>2</sub>.

necessitating the use of an average in each experiment to determine the per cent recovery of the added glucose. Also, from a comparison of these two columns it can be seen that the lead subacetate removes variable amounts of the reducing material,

TABLE 1

*Reducing sugar in medium, as determined by the Shaffer-Hartmann method after clarification with lead subacetate*

(Four duplicates were run on each sample; the extremes and averages are given below)

(1)	(2)	(3)	(4)	(5)	(6)
DATE	REDUCING SUGAR IN MEDIUM WITHOUT CLARIFICATION	REDUCING SUGAR AFTER CLARIFI- CATION WITH Pb SUBACETATE	LOSS IN REDUC- ING SUGAR DUE TO CLARIFICA- TION	REDUCING SUGAR IN MEDIUM + 100 MGM. GLUCOSE, LEAD SUBACE- TATE TREATMENT	RECOVERY OF ADDED GLUCOSE
	<i>mgm.</i>	<i>mgm.</i>	<i>per cent</i>	<i>mgm.</i>	<i>per cent</i>
7/17	162-163 Av. 162	134-141 Av. 136	10	174-177 Av. 175	39
7/28	148-150 Av. 150	134-139 Av. 137	9	226-235 Av. 230	90
7/28a	146-150 Av. 147	78- 82 Av. 81	45	150-152 Av. 151	70
7/29	145-187 Av. 170	148-159 Av. 154	9	250-261 Av. 257	103
8/2	162-164 Av. 163	153-159 Av. 157	3	242-256 Av. 250	93
8/2a	159-193 Av. 177	136-155 Av. 146	16	245-249 Av. 247	101
11/10	108-122 Av. 115	93-117 Av. 105	10	177-188 Av. 185	80
11/11	120-120 Av. 120	147-148 Av. 148	20*	241-242 Av. 242	94

\* This was a gain of 20 per cent.

from 3 to 45 per cent of that present in the original medium. With this method of precipitation there was a recovery of added glucose within the experimental error (plus or minus 4 per cent) in only two of the eight experiments. The averages of experi-

TABLE 2

*Reducing sugar in medium, determined by the Shaffer-Hartman method without previous clarification*

(Four duplicates were run on each sample; the extremes and averages are given below)

(1) DATE	(2) RECOVERY OF 100 MGM. OF GLUCOSE	(3) REDUCING SUGAR IN MEDIUM	(4) REDUCING SUGAR IN MEDIUM PLUS 100 MGM. OF ADDED GLUCOSE	(5) RECOVERY OF ADDED GLUCOSE
	<i>mgm.</i>	<i>mgm.</i>	<i>mgm.</i>	<i>per cent</i>
7/27	101 101	163-170 Av. 166	258-267 Av. 263	97
7/27a	101 101	158-159 Av. 158	260-264 Av. 262	104
7/28		148-150 Av. 150	247-249 Av. 247	97
7/28a		146-150 Av. 147	253-259 Av. 255	108
8/2		159-193 Av. 179	285-317 Av. 301	124
8/2a		162-164 Av. 163	254-279 Av. 270	107
7/29	96 96	145-187 Av. 170	272-299 Av. 289	119
9/21	98 99	134-140 Av. 136	230-239 Av. 234	98
9/22		130-136 Av. 133	234-236 Av. 235	102
9/22a		120-138 Av. 131	230-233 Av. 231	100
9/30	99 96	140-145 Av. 142	233-234 Av. 233	91
10/28	97 97	139-151 Av. 142	227-240 Av. 232	90
11/10	99 100	107-122 Av. 115	119-204 Av. 202	87
11/11	100 100	120-120 Av. 120	173-176 Av. 175	55

ments 7/29 and 8/2a indicate an acceptable recovery of glucose, but only one (7/29) is satisfactory since in 8/2a the variation between the duplicates is 6 per cent, which exceeds the experimental error. If this may be considered a true index of accuracy, there is only one sample out of the eight which gives a complete recovery of glucose.

Since the lead subacetate removed variable amounts of the reducing material and the recovery of glucose was inaccurate, an attempt was made to apply the Shaffer-Hartmann method directly to the medium without previous clarification, the second method suggested by Stiles, Peterson and Fred. The data obtained by the use of this procedure appear in table 2.

With this method the recoveries of added glucose vary from 55 to 124 per cent (column 5). Six of them are within the experimental error and of the six there is only one (9/22a) whose variations in the original sugar determinations (column 3) are not acceptable. Therefore, with this method there were five accurate recoveries in the eight samples analyzed, which is somewhat more successful than the result obtained by the lead subacetate method.

From these determinations it would appear that the application of the Shaffer-Hartman method to media without preliminary treatment is the more reliable procedure, but the irregularities are considerable and without obvious explanation. These irregularities might cast doubt on the technique used in carrying out the determinations, but the estimations of glucose in a standard solution (column 2, table 2) possess all the uniformity that could be desired. These check determinations were made simultaneously with those upon the medium, as indicated by the dates in the tables.

Since the lead clarification method was found to be unsuccessful, some other methods of precipitation were investigated. The first to be used was the Folin-Wu precipitation with tungstic acid. In one series of experiments the same dilution was used as in the preparation of blood filtrates, 1 to 10, but this procedure gave no better results. The amount of reducing material removed by the precipitant varied from 7 to 24 per cent, and the recovery of added glucose was from 91 to 118 per cent. Since

the meat infusion broth does not contain as much protein as does blood, a 1 to 20 dilution of the tungstic acid and medium was attempted. Again the method was found to be useless, for the precipitant removed from 1 to 30 per cent of the reducing material in the untreated medium.

Other protein precipitants were tried with similar results. With phosphotungstic acid as a precipitant, 61 to 76 per cent recoveries of added glucose were obtained. Mercuric nitrate, used as described by Ronzoni and Wallen-Lawrence (1927), gave a biuret-free filtrate, the only one we were able to obtain with any of the methods of precipitation, but the recoveries of added glucose were just as variable as before, ranging from 64 to 120 per cent, and none were within the limits of experimental error.

Since the use of the Shaffer-Hartmann method for the determination of reducing sugar in media was not satisfactory, Benedict's colorimetric method (1926) was considered for this purpose. A slight modification of the method was employed; Benedict's uric acid reagent was used instead of the color reagent described for the original sugar method, since this modification gives a more easily matched color. With this method, theoretical values were obtained with standard solutions equivalent to 100 and 200 mgm. of glucose. Duplicate determinations were always made and the recovery experiments were controlled, as in the previous procedures. This method was used on media without preliminary treatment, and also after the Folin-Wu and lead subacetate methods of precipitation. The results are given in table 3.

Again our results were unsatisfactory. The variations between the duplicate sample and the range of percentage recoveries are almost as great as those obtained with the Shaffer-Hartmann method. Of seven samples of the media which had not been clarified (column 5) only one accurate recovery was obtained, the recoveries ranging from 86 to 119 per cent. With the Folin-Wu precipitation (column 8), we obtained satisfactory recoveries in three out of seven determinations, but in only one of these (12/8) are the averages sufficiently close to be considered accurate. The results after the use of lead subacetate are just as variable; in six determinations we obtained but one accurate recovery

(12/10). With such results we feel justified in discarding Benedict's method.

TABLE 3  
*Reducing substances in media, determined by Benedict's colorimetric method*

DATE	RECOVERY OF 100 MG. GLUCOSE	NO PRECIPITATION			FOLIN-WU FILTRATE			LEAD SUBACETATE PRE- CIPITATE		
		Reducing substance	Medium + 100 mgm. glucose	Recovery	Reducing substance	Medium + 100 mgm. glucose	Recovery	Reducing substance	Medium + 100 mgm. glucose	Recovery
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
	mg.	mg.	mg.	per cent	mg.	mg.	per cent	mg.	mg.	per cent
12/8		141	230		128	227				
		141	236		122	218				
		Av. 141	Av. 233	92	Av. 125	Av. 223	98			
12/8a		105	198		115	206		105	183	
		109	206		105	198		115	203	
		Av. 107	Av. 202	95	Av. 110	Av. 202	92	Av. 110	Av. 193	83
12/9	101	120	204		103	191		116	210	
	100	119	206		111	200		119	206	
	202*	Av. 119	Av. 205	86	Av. 107	Av. 195	88	Av. 117	Av. 208	91
	202*									
12/10	98	125	222		125	220		111	211	
		128	218		125	218		120	211	
		Av. 126	Av. 220	94	Av. 125	Av. 219	94	Av. 115	Av. 211	96
12/11	100	122	224		110	200		107	213	
		120	256		95	198		110	244	
		Av. 121	Av. 240	119	Av. 102	Av. 199	97	Av. 108	Av. 228	120
12/16	101	132	226		118	235		129	197	
		135	221		131	214		132	210	
		Av. 133	Av. 223	90	Av. 125	Av. 225	100	Av. 130	Av. 204	76
12/16a		117	211		122	224		130	240	
		125	235		115	224		113	238	
		Av. 121	Av. 223	102	Av. 119	Av. 224	105	Av. 124	Av. 239	115

\* Recovery of 200 mgm. glucose.

Attempts to determine if the variable factor was due to the peptone or the beef extractives were unsuccessful, because the Shaffer-Hartmann method is not adapted to the determination



of such small amounts of reducing material as are found in peptone. From 80 to 90 per cent of the reducing material was found to come from the water-soluble constituents of the beef.

By means of yeast fermentation (Benedict 1928) it was found that approximately 40 per cent of the total copper reducing material consisted of non-glucose reducing substances.

#### CONCLUSIONS

Reducing sugar in bacteriological media could not be determined accurately by the Shaffer-Hartmann method as used by Stiles, Peterson and Fred on filtrates obtained by treating the medium with lead subacetate.

When other protein precipitants were used the results were equally unsatisfactory. Tungstic acid in two different dilutions, phosphotungstic acid and mercuric nitrate were tried as precipitating agents.

Benedict's colorimetric method was found to be unsatisfactory, since it was found to be impossible to recover added quantities of glucose consistently.

The least variable results were obtained when the Shaffer-Hartmann method was applied to media without previous clarification.

Since meat infusion broth is used more frequently than any other medium for general bacteriological work, it would appear that workers interested in the sugar content of such media must be satisfied, for the present, with micro sugar methods whose results have wide limits of accuracy, certainly not less than plus or minus ten per cent.

The authors wish to express their gratitude to Professor Henry A. Mattill of this department for his valuable suggestions and coöperation in this work.

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# COMPOUND FORMATION OF CRYSTAL VIOLET WITH NUCLEIC ACID AND GELATIN AND ITS SIGNIFICANCE IN DYE BACTERIOSTASIS

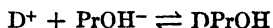
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In 1924, Stearn and Stearn outlined a chemical theory of the mechanism of bacteriostasis by dyes, based on the amphoteric behavior of bacteria. A system of equilibria was set up to explain the pH effect on limiting dye dilutions, as well as the fact that inhibiting action is often not bactericidal. This set of postulated equilibria indicated that, with basic bacteriostats such as the triphenylmethane dyes, the more highly ionized the dye base, the more effective it will be. In a general way the relation between ionization and toxicity of strong acids and bases was established by Krönig and Paul (1897), but an intriguing question arose as to why certain basic ions such as triphenylmethane dyes were effective as bacteriostats at such high dilutions whereas other basic ions such as potassium or sodium were not.

This involves a consideration of the equilibrium



where  $D^+$  represents the bacteriostatic cation and  $\text{PrOH}^-$  any anion of the bacterium which is affected by the bacteriostat. If the resulting salt,  $\text{DPrOH}$ , is itself highly ionized, one would expect little or no bacteriostatic action on the postulated chemical mechanism. On the other hand, if there are cations which tend to form co-valent bonds, i.e., un-ionized valance linkages, with vital components of the bacterium, then, small amounts of such cations should be bacteriostatically effective.

The present paper describes some experiments carried out to study this ionization equilibrium in the case of crystal violet

as cation, with gelatin as a type protein and yeast nucleic acid as a type nucleic acid. Such substances are typical of some of the more important components of the bacterial cell which might be expected to combine, under proper conditions, with cations.

The method used was straightforward. If the conductivity of the crystal violet ion in one solution is known and that of nucleate ion or gelatinate ion in another solution is known, then, if known volumes of these solutions are mixed, the conductivity of the mixture should be easily calculable provided no combination between any of the ions has taken place. A decrease in conductivity from that calculated would indicate such combination.

The nucleic acid was an Eastman product. When ignited it gave a glassy residue of 6.6 per cent. This residue gave only the faintest traces of chlorides or sulfates and consisted of pyrophosphate from the nucleic acid. Inasmuch as the solutions used were mixtures of the free nucleic acid and its salt due to pH adjustment this preparation could be considered free from any impurity which would affect the results. The gelatin was ash-free, containing, on ignition 0.08 per cent residue. The crystal violet was a product of the National Aniline Company certified by the Commission on the Standardization of Biological Stains. It gave, on ignition, only 0.26 per cent residue. In this work the stock solutions, made up fresh as required and, except for the dye solutions, always used the same day they were made up, were 1 per cent gelatin, 1.5 grams per liter of the crystal violet, and 0.5 per cent nucleic acid. Pure nucleic acid is not soluble to this extent but since runs were made only to a pH of 3.9, enough NaOH was added to bring the pH to this value and at such a reaction a 0.5 per cent solution can be obtained. Except for a few preliminary runs to study the effect of KCl on the various reagents, the temperature was maintained at  $28.5^{\circ}\text{C} \pm 0.05^{\circ}$ .

#### EXPERIMENTAL

##### *Preliminary work with KCl*

The following preliminary experiments show that calculated and measured conductivity values agree within 1 to 2 per cent

when KCl is used with either the dye, the gelatin or the nucleic acid. These were made at 25°C. The unit of conductivity is for convenience taken as  $10^{-6}$  reciprocal ohms.

- I. 50 cc. KCl solution increased the conductivity of 500 cc. water by 89.4 units.  
50 cc. KCl solution increased the conductivity of 450 cc. water and 50 cc. dye solution by 88.5 units. (Diff. 1.0 per cent.)  
50 cc. KCl solution increased the conductivity of 475 cc. water and 25 cc. nucleic acid (pH 5.1) by 88.1 units. (Diff. 1.45 per cent.)
- II. 25 cc. nucleic acid solution (pH 5.1) increased the conductivity of 500 cc. water by 57.8 units.  
25 cc. nucleic acid (pH 5.1) increased the conductivity of 450 cc. water and 50 cc. KCl solution by 57.5 units. (Diff. 0.5 per cent.)
- III. 10 cc. of KCl solution increased the conductivity of 350 cc. water by 139.5 units.  
10 cc. this KCl solution increased the conductivity of 250 cc. water and 100 cc. isoelectric gelatin by 140 units. (Diff. 0.4 per cent.)  
10 cc. this KCl solution increased the conductivity of 250 cc. water and 100 cc. gelatin (pH 7) by 138 units. (Diff. 1.08 per cent.)

### *Method*

When a solution of crystal violet adjusted to any particular pH below a certain limit is mixed with one of gelatin or of nucleic acid adjusted to the same pH, there is invariably a decrease in pH. In solutions of low hydrogen ion concentration this change does not appreciably affect the conductivity values from the increase of hydrogen ions, but at higher hydrogen ion concentrations it may seriously affect them. For this reason, though these systems were studied through a wide pH range, the results here presented are confined to values obtained between the pH limits of 5.5 to 7, and, for the data given, the effect due to pH change in no case amounted to more than 3 per cent of the total effect, usually to a much lower per cent. The significance of this pH change for our ideas of the physical chemistry of proteins will be discussed elsewhere.

*Procedure*

The ordinary procedure was as follows: 500 cc. of water was brought to the proper temperature and pH. Inasmuch as the work at lower pH required adjustment of the distilled water itself before starting a run, there would have been no point in preparing special conductivity water. A good grade of distilled water was used whose conductivity, before pH adjustment, averaged 4 to 6 by  $10^{-6}$  reciprocal ohms with the cell employed. pH adjustment of the water was made where necessary, and in all cases the conductivity of the water blank corrected for in making the subsequent calculations.

If, for example, the system dye-nucleic acid was being studied, increments of the nucleic acid adjusted to the pH of the water blank were added to this 500 cc. of water and the conductivity of the resulting solution determined after each addition. When this process had been completed, the solution was replaced by 500 cc. of a mixture of stock dye solution and water at the proper pH. Its conductivity was determined and then increments of the nucleic acid solution were added as in the case of the blank with conductivity determinations between each addition. All values were then corrected for volume change.

*Calculations*

From the above data, the conductivity due to a certain quantity of nucleic acid in water and in dye solution could be compared. To obtain a semi-quantitative idea of the reaction between the two ions, the decrease in conductivity of the nucleic acid due to the presence of dye was divided by the sum of the conductivities of nucleate ion and dye ion in solutions which had not been mixed. These latter can be obtained either by subtracting the conductivity of sodium ion (from the NaOH used in pH adjustment) and chloride ion of the dye salt from the total conductivities of these reagents, or, more easily and in some cases more precisely, they may be obtained from the transference numbers of the dye ion in the chloride and the nucleate ion in its sodium salt.

The latter method has the advantage for the nucleate ion that the presence of a certain amount of salt in the original

preparation of nucleic acid will not affect the results. From a study of a large number of related compounds (Bredig, 1894) the mobility of the dye ion was taken as about 24 to 25 at 25°C., giving it a transference number in the chloride of about 0.25. Calculations made on the data of the gelatin solutions with the

TABLE 1  
*System: Dye—nucleic acid—pH 5.5*

NUCLEIC ACID SOLUTION ADDED	500 CC. WATER + NUCLEIC ACID K <sub>1</sub>	450 CC. WATER AND 50 CC. DYE + NUCLEIC ACID K <sub>2</sub>	CONDUCTIVITY DECREASE -ΔK	CONDUCTIVITY OF DYE AND OF NUCLEATE ION BEFORE MIXING K	DECREASE
cc.					per cent
0		57 45			
5	12 22	62 83	6.8	18 3	37 0
10	27 13	69 9	14 7	23 1	63 6
15	42 75	78.27	21 9	28 4	77.0
20	58 55	88 0	28 0	33 1	84 5
25	74 0	102.4	29 0	38 1	76 0
30	89 5	115 1	31.9	43 0	74 0
35	105 7	131.5	31 7	48 2	66.0

The values in column 4 are obtained from the formula  $57.45 + K_1 - K_2$ . Those in column 5 are obtained from the formula  $57.45 \times$  transference number of dye ion in the chloride plus  $K_1 \times$  transference number of nucleate ion in the sodium salt.

TABLE 2  
*System: Dye—nucleic acid—pH 5.5—300 cc. water plus 200 cc. dye solution*

NUCLEIC ACID SOLUTION ADDED	DECREASE IN CONDUCTIVITY OF DYE AND NUCLEATE IONS
cc.	per cent
25	56 8
45	78 5
65	87 5
85	86 5

known amounts of NaOH added gave a transference number of gelatinate ion of about 0.3, a value in accord with the general findings of Pauli (1922) on the mobility of protein ions. The mobility of nucleate ion was taken as 25 corresponding to an anionic transference number of about 0.32.

*Results*

Table 1 gives an idea of the data and calculations. Conductivity values, in columns labeled K, are in units of  $10^{-6}$  reciprocal ohms. The conductivities have been corrected for the water

TABLE 3  
*System: Dye—nucleic acid—pH about 7*

A. 450 CC. WATER AND 50 CC. DYE SOLUTION		B. 400 CC. WATER AND 100 CC. DYE SOLUTION		C. 300 CC. WATER AND 200 CC. DYE SOLUTION	
Nucleic acid added	Conductivity decrease	Nucleic acid added	Conductivity decrease	Nucleic acid added	Conductivity decrease
cc.	per cent	cc.	per cent	cc.	per cent
5	58.4	5	25.0	25	60.5
10	71.4	10	45.2	45	77.5
15	82.0	15	61.5	65	77.1
20	80.6	20	73.3	85	67.0
25	74.0	25	80.5		
		30	82.3		
		35	80.5		

TABLE 4  
*System: Dye—gelatin—pH 5.5*

A. 450 CC. WATER AND 50 CC. GELATIN		B. 300 CC. WATER AND 200 CC. DYE SOLUTION	
Dye solution added	Conductivity decrease	Gelatin solution added	Conductivity decrease
cc.	per cent	cc.	per cent
20	18.0	20	9.0
40	29.0	40	17.0
60	30.5	60	23.5
80	29.0	80	29.3
100	28.5	100	33.5

TABLE 5  
*System: Dye—gelatin—pH about 7*

A. 450 CC. WATER AND 50 CC. GELATIN		B. 300 CC. WATER AND 200 CC. DYE SOLUTION	
Dye solution added	Conductivity decrease	Gelatin solution added	Conductivity decrease
cc.	per cent	cc.	per cent
20	17.0	20	13.6
40	30.8	40	24.2
60	35.3	60	33.0
80	37.0	80	40.0
100	32.0	100	46.0

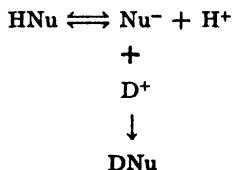
blank and volume change resulting from the addition of the titrating solution. The temperature was 28.5°C.

A digest of more results is given in tables 2 to 5.

#### DISCUSSION

The types of systems studied permit only a qualitative interpretation of the data given. It is thought, however, that the magnitude of the effect is sufficient to consider it as distinctly confirmatory of a chemical mechanism for this type of bacteriostasis, unless it be argued that the properties of gelatin and of yeast nucleic acid are highly specific, and do not represent general behavior.

The curves obtainable from these data can be easily seen to be of the form typical of conductivimetric titration curves with fairly definite maxima. At first sight it might be argued that the conductivity decreases may be entirely due to mutual colloidal flocculation of oppositely charged particles, and not to chemical effects. Two kinds of observations are, however, distinctly opposed to such a view. In the first place the observed decrease in conductivity is independent of whether or not there is accompanying precipitation of dye-gelatin or dye-nucleate. Under some conditions such precipitation occurs, but under others it does not and the order of magnitude of the conductivity decrease is the same in both cases. In the second place the pH change when dye and nucleic acid or gelatin solutions are mixed is a typical chemical replacement reaction. Thus, if a solution containing nucleate ion and some unneutralized nucleic acid adjusted to a certain pH be added to one containing dye ion at the same pH, the conductivity data above given indicate that the following reactions take place,



where Nu stands for the nucleate radicle and D for the dye radicle. The dye ion removes nucleate ion forming unionized dye



nucleate and causing more nucleic acid to ionize in maintaining its ionic equilibrium, with a resulting increase in hydrogen ion concentration. Typical data showing this effect are given in table 6. Here, to 50 cc. of dye solution at a pH of 5.62, were added increments of nucleic acid solution at a pH of 5.71.

The data in tables 1 to 5 can be put in much more striking form. As they stand they do not show the effect of an excess

TABLE 6

NUCLEIC ACID, pH 5.71, ADDED TO 50 CC. DYE, pH 5.62	pH RESULTING
cc.	
5	4.93
10	4.80
15	4.93
20	5.35
25	5.50
30	5.55
40	5.58
50	5.62

TABLE 7

450 cc. water and 50 cc. dye solution—pH 5.5

NUCLEIC ACID ADDED	K (NUCLEATE ION)	K (NUCLEATE ION BLANK)	CONDUCTIVITY DECREASE
cc.			per cent
5	3.4	3.9	87.0
10	7.35	8.7	85.0
15	10.95	13.7	80.0
20	14.0	18.7	75.0
25	14.5	23.7	61.0
30	15.95	28.6	56.0
35	15.85	33.9	47.0

of dye on the "disappearance" of nucleate or gelatinate ion from solution. They merely indicate total effects distributed between both ionic species. One can easily bring out the order of magnitude of the effect of excess dye as follows: It was seen above that the respective mobilities of dye ion, gelatinate ion and nucleate ion are probably not very different. If, then, we split up the total decrease in conductivity (i.e., the data analogous to those in the

column labelled  $\Delta K$  in table 1) equally between the two ions involved, the effect on the ion present in smaller concentration can be calculated. Thus the nucleate ion would account for half the decrease in conductivity (table 1) and this half, in place of being referred to the sum of the blank conductivities of both ions as in the column 5, table 1, is referred only to that of the nucleate ion, i.e., a value calculated from  $K_1$  (table 1) times the transference number of nucleate ion in the sodium salt. The data of table 1 so calculated are given in table 7.

TABLE 8  
*System: Dye—nucleic acid*

pH 5.5, 300 CC. WATER AND 200 CC. DYE		pH 7, 450 CC. WATER AND 50 CC. DYE		pH 7, 400 CC. WATER AND 100 CC. DYE		pH 7, 300 CC. WATER AND 200 CC. DYE	
Nucleic acid added	Conduc- tivity decrease	Nucleic acid added	Conduc- tivity decrease	Nucleic acid added	Conduc- tivity decrease	Nucleic acid added	Conduc- tivity decrease
cc.	per cent	cc.	per cent	cc.	per cent	cc.	per cent
25	101 0	5	100 0	5	97 5	25	96 5
45	94.5	10	81 0	10	89 5	45	86 0
65	87.0	15	74 0	15	88 0	65	69.5
85	76 0	20	65 0	20	87.0	85	55 5
		25	55.0	25	84 3		
				30	78 5		
				35	72.0		

Values calculated in an analogous manner from the data of tables 2 to 5 are given in tables 8 and 9.

The above results indicate that, in the presence of an excess of dye, gelatinate or nucleate ion is practically completely combined through a non-ionized linkage. The results in tables 1 to 5 show that the excess does not have to be enormous. It is thought that these data may throw some light on the problem of the effective bacteriostatic action of triphenylmethane dyes at high dilution. When compared stoichiometrically it has been shown (Stearn and Stearn, 1924) that even 1:10,000,000 of crystal violet in broth gives nearly 100 equivalents of dye to one of bacterial anion with an inoculum of 4000 organisms in 5 cc.

Recently Dubos (1929) has suggested that the effect of dyes in

inhibiting the growth of organisms may be due to their maintaining an increased, and therefore adverse, oxidation potential in the medium. This idea is supported by his statement that the reduced dyes lack much of this inhibiting power. While the importance of the oxidation-reduction environment of a medium is a factor of prime importance and should merit consideration in the mechanism of the dye bacteriostasis if it is shown that this environment is affected by the dyes used, nevertheless it is difficult for the present author to imagine any significant change

TABLE 9  
*System: Dye—gelatin*

pH 5.5, 300 CC. WATER AND 200 CC. DYE SOLUTION		pH 7, 300 CC. WATER AND 200 CC. DYE SOLUTION	
Gelatin solution added	Conductivity decrease	Gelatin solution added	Conductivity decrease
cc.	per cent	cc.	per cent
20	133.0*	20	105 0
40	120 0	40	101 0
60	109 0	60	100.0
80	107.0	80	95 5
100	101 0	100	92 5

\* These high values are due to the fact that a pH of 5.5 is at a point in the titration curve of gelatin where small pH changes are equivalent to large changes in gelatinate ion concentration. The effect noted in the data of table 6 comes into play here with large excess of dye. Thus, all the original gelatinate ion is removed and more is formed by the shift in the ionization equilibrium of gelatin and this, in turn, is removed by the large excess of dye.

in the oxidation-reduction potential arising from 1:60,000,000 of crystal violet against the "poising" action of ordinary medium constituents (Stearn and Stearn, 1928) or even from a concentration of 1:1,000,000, where many organisms are affected.

Another point which should be considered in this connection is the fact that the efficiency of these dyes has been shown to increase with increasing pH (Stearn and Stearn, 1926), whereas, for constant ratio of color dye to its reduced form, the oxidation potential, if it changes at all with pH, should *decrease* instead of increasing with increasing pH.

For these reasons some other mechanism should be sought for

this bacteriostatic action, and the results of this paper strongly support the idea of co-valent linkages between the dye ion and the various anions of the bacterium such as those of the proteins, the nucleic acids, etc., thus "binding" them and preventing their normal physiological functioning.

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# A SUGAR-TOLERANT MEMBER OF THE COLON-AEROGENES GROUP

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Investigations of the decomposition of raw sugars have demonstrated the ability of microorganisms of various types to grow in concentrated sugar solutions. Owen (1911) and other workers showed that certain members of the aerobic spore-forming group, capable of growth in high sugar concentrations, were common in sugar refineries and were responsible for the decomposition of raw sugars and sugar products. While studying the deterioration of cane sugar in Louisiana, Kopeloff and Kopeloff (1919) isolated highly sugar-tolerant<sup>1</sup> molds which were responsible for certain types of sugar deterioration in storage.

In 1918 Browne isolated strains of torula, monilia, and a rod-shaped bacterium from raw sugars. He found these microorganisms were the most common forms present in Cuban raw sugars. Other organisms were found also, but were not studied. He concluded that no one type of microorganism alone was responsible for the deterioration of commercial raw sugars, but that bacteria, molds, and budding fungi should be suspected. He concluded further that these microorganisms were unable to grow in saturated solutions and suggested the remedy which sugar producers have since found effective in preventing deterioration losses.

<sup>1</sup> In describing the characteristics of microorganisms, careful distinction should be made between the words "resistant" and "tolerant." The former should be used in considering the ability of an organism to combat conditions which are tending to destroy it; while "tolerant" should be used to describe the ability of an organism not only to resist outside injurious influences, but also to be capable of growth or metabolism under the conditions in question.

Shutt (1925) in studying the bursting of chocolate-coated creams found that yeast present in a candy factory grew and produced gas in commercial fondant, the filling for chocolate-coated creams.

While studying in the Department of Agriculture the relation of sugar-tolerant organisms to certain industrial practices, Church, Paine and Hamilton (1927) isolated sugar-tolerant yeasts and demonstrated their relation to the fermentation of fondant. They concluded that these microorganisms were responsible for the bursting of chocolate-coated cream candies.

From these and many other studies, it is evident that strains of organisms capable of growth in sugar solutions of rather high density may be encountered among the bacteria, yeasts, or molds. The further studies of this nature are carried the more we are forced to believe that highly sugar-tolerant organisms, highly salt-tolerant organisms, and highly heat-tolerant organisms are widely distributed in nature. Knowledge of the biochemical phenomena involved in the metabolism of these organisms, however, is woefully lacking.

Following the isolation of spoilage thermophiles from sugar (Cameron, Williams and Thompson, 1927, and James, 1928a), a survey of the microbial flora of commercial sugars was made in this laboratory (James, 1928b). In this survey the results of many of the tests for putrefactive anaerobes were unsatisfactory because of a liberation of gas in the anaerobic media to which the original test sugar solution had been added, but in which putrefactive anaerobes could not subsequently be found. This indicated the presence of organisms capable of growing under anaerobic conditions only in the presence of fairly large concentrations of sucrose. This conclusion was supported by the occasional development of gas bubbles in deep agar tubes containing 10 per cent sucrose, which were run on a few samples to test for sugar-tolerant anaerobes. Some time after the survey had been completed, some of the original anaerobic tubes were removed from cold storage, and further studies of their microbial contents were made.

Time did not permit a study of more than a few of the tubes,

and most of the organisms have died since. In the course of the tests, however, an organism which possessed interesting characteristics was isolated from a deep tube of Kopeloff's agar containing 10 per cent sucrose. It produced abundant gas within twenty-four hours, incubation at 37°C.

Cultural studies of this organism have shown it to be a Gram-negative, non-spore-forming, short, plump rod. It grows best under aerobic conditions, but in the presence of sugar grows well anaerobically. It is actively motile, ferments lactose with acid and gas production, and, as judged by its cultural and morphological characteristics, belongs to the species designated by Weldin (1927) as *Aerobacter cloacae*. We do not consider it a new species of the colon-typhoid group, but only one which through long exposure to adverse conditions has acclimated itself to its surroundings, until now it can grow anaerobically in fairly concentrated sugar solutions. It produces abundant gas in both liquid and solid media containing sucrose in concentrations from 1 to 30° Brix. In a 35° solution its growth is not as luxurious as in the lower concentrations, but it still shows considerable development. Gelatin is not liquefied.

In considering the tolerance of microorganisms in concentrated solutions, careful attention should be given to the methods of preparation of the test solutions and to the treatments accorded the microorganisms. Tests of an organism's ability to do certain things cannot be fairly made unless it is given every opportunity to adapt itself to the conditions. Inoculations into media containing 20, 40, 60 or more degrees Brix of sucrose should not be made from ordinary broth or agar cultures. The highest concentration showing growth should be used as inoculum, and, then, not more of the culture than necessary should be added, in order to avoid diluting the test medium more than a fraction of a degree Brix. Media containing more than the usual 1 per cent of sugar should always be controlled by saccharometer or polariscope readings, and not made up by weight or volume alone.

#### SUMMARY

An actively motile, sugar-tolerant organism resembling *Aerobacter cloacae* has been isolated from granulated cane sugar. It



actively ferments sucrose in a concentration of 30° Brix, shows delayed fermentation of sucrose in a concentration of 35° Brix, and does not ferment or grow in a concentration of 40° Brix.

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# AN EXPLANATION OF THE ACTION OF THE SO-CALLED ACCESSORY SUBSTANCES IN THE ASSOCIATION OF AZOTOBACTER AND CELLULOSE DECOMPOSING ORGANISMS

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## INTRODUCTION

The brilliant researches in the field of vitamins in animal nutrition have led many investigators to study the effect of accessory factors on growth of green plants, yeasts, and bacteria. Although positive results have been claimed, critical study of the literature will disclose the fact that, as yet, no organic substance is known to be essential to normal healthy growth of green plants grown in properly controlled mineral nutrient solutions. The so-called "auximones" of Bottomley (1914 a, b) can no longer be accepted and the term should be dropped (Wolfe, 1926). Likewise, the work of Fulmer and associates (1922, 1923, a, b), Tanner and associates (Tanner, 1924, Wallace and Tanner, 1928), and Werkman (1925) have demonstrated that "bios," supposedly essential for the growth of yeasts, is not essential, and merely acts as a favorable source of organic nitrogen or other food. Werkman's work was particularly rigid. He isolated several strains of yeasts by single cell technique, grew them in a very carefully purified mineral salt solution, with a synthetic sugar as the only organic compound, and transferred them into fresh media every two or three days for five months, at the end of which time they were still viable. There has been little critical work on the necessity of accessory factors for the growth of bacteria. While it is known that certain bacteria, such as the Pfeiffer bacillus, need minute amounts of blood or vegetable tissue for normal development,

yet it is a common experience that many bacteria grow well in purely synthetic media.

Recent publications of Sanborn (1926 a, b, 1927), in which the terms "accessory," "stimulating" and "essential" are apparently used interchangeably, have appeared, and the data contained therein are interpreted by their compiler as indicating that certain substances found in vegetable extracts, soil, and the metabolic products of *Azotobacter chroococcum* and other bacteria are essential, stimulating, or accessory to, the "physiological efficiency" of a cellulose decomposing bacterium. Essential the substances are not, as shown by Sanborn's own data. Stimulating to growth they are, but, as shown by Werkman (1927), they do not act as accessory substances in the way that vitamins act on animal growth as implied by Sanborn, but merely as readily available organic food. Werkman showed this to be true not only of Sanborn's data but also of those of Itano (1923) on "accessory" substances in the nutrition of *Azotobacter* itself. Werkman, however, while explaining the increased growth in terms other than those implying accessory substances, did not attempt to explain what Sanborn called "physiological efficiency," nor did he do any work on the elaboration of these "essential substances" in associative action.

"Physiological efficiency," as I understand it from Sanborn's papers, means cellulose-decomposing ability as measured by increase in acidity. Whether the acidity is due to the acid intermediate products of cellulose decomposition, or to the freeing of the  $\text{SO}_4$  ion from the ammonium sulphate in the media, due to the absorption of the  $\text{NH}_4$  radical by the organism, Sanborn did not state. The "physiological acidity" of ammonium salts is well known to plant physiologists, agronomists, and bacteriologists.

I propose to subject Sanborn's data and other data accumulated by myself in the past thirty months to critical analysis and see if a better explanation for the results may not be deduced. Certainly an explanation depending upon the known physiology of organisms is preferable to one based upon a hypothetical substance, whose mode of action (if the substance exists) is a mystery. This is especially desirable when we consider the fate of the terms "auximone," "bios," "allelocatalytic substances," etc.

Unfortunately, I could not use the organism that Sanborn worked with as I was unable to obtain a culture from him. Instead, I used four organisms, two of which are well known and fairly easily isolated, and the identical strains of three of which may probably be obtained. They are *Spirochaeta cytophaga* from P. H. H. Gray of Rothamsted, England, *Trichoderma kőningi*<sup>1</sup> from Professor Waksman of Rutgers University and the "Y" organism from Dr. Dubos (1928), now of the Rockefeller Institute. I also used a culture of sterile mycelium isolated by myself from soil, which unfortunately has since died out. Although I hesitate to use the data obtained by means of a culture which, because of its nature, cannot be described, and which is no longer obtainable, I include them since they merely corroborate data of Sanborn and myself. The "associative" organism was a strain of *Azotobacter chroococcum* isolated by Beijerinck and cultivated by Dean Lipman of Rutgers University for more than a quarter of century.

#### EXPERIMENTAL

The basic medium used by Sanborn was as follows:

K <sub>2</sub> HPO <sub>4</sub> .....	1 gram
MgSO <sub>4</sub> (anhydrous) .....	1 gram
Na <sub>2</sub> CO <sub>3</sub> (anhydrous) .....	1 gram
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> .....	2 grams
H <sub>2</sub> O (distilled) .....	1000 cc.

This is a modification of the well known solution of McBeth and Scales (1913) who added an excess of CaCO<sub>3</sub>. It was found that some of the organisms did not grow in either of the media due to the extremely high pH. Therefore, McBeth's and Scales' solution was modified by omitting the CaCO<sub>3</sub> and substituting NaCl for Na<sub>2</sub>CO<sub>3</sub>. In certain cases the reaction was changed further by the addition of NaOH or HCl. All four of my organisms grew well in this solution with filter paper as the source of energy. In some instances, to be explained later, an equivalent amount of KNO<sub>3</sub> was substituted for the (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. In every case the

<sup>1</sup> *Trichoderma* sp. used by Heukelekian and Waksman\* (1925) was identified as *T. kőningi*.

sterilized nitrogen compound was added aseptically after sterilization of the medium.

As a source of "cellulose," Sanborn used filter paper in a few instances, but usually "unginned and chemically-untreated" raw cotton. Since he made no chemical analyses to determine the amount of cellulose decomposed, but depended upon the change in the pH, it is obvious that he may have been measuring not cellulose decomposition, but the decomposition of any one or many of the other compounds in raw and unginned cotton. Very small changes in acidity would register as large changes in pH in a

TABLE 1

*The effect of Azotobacter chroococcum on the reaction change and decomposition of cellulose by Trichoderma kőningi in solutions containing ammonium and nitrate salts (6 days' incubation)*

FLASK NUMBER	INOCULATION	SOURCE OF NITROGEN	TITRATION, STANDARD KMnO <sub>4</sub> FOR ALiquOT USE	pH
			cc.	
1	Control	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	17.1	6.67
2	Trichoderma	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	17.0	3.15
3	Trichoderma	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	17.3	3.09
4	Trichoderma and Azotobacter	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	17.2	2.82
5	Trichoderma and Azotobacter	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	17.2	2.92
6	Control	KNO <sub>3</sub>	17.2	6.88
7	Trichoderma	KNO <sub>3</sub>	17.2	6.95
8	Trichoderma	KNO <sub>3</sub>	17.2	7.16
9	Trichoderma and Azotobacter	KNO <sub>3</sub>	17.1	7.08
10	Trichoderma and Azotobacter	KNO <sub>3</sub>	17.3	7.44

medium so poorly buffered. For my experiments Eaton and Dikeman No. 615 filter paper was used.

Ten flasks each containing 100 cc. of medium (five with KNO<sub>3</sub> and five with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) were prepared with equal amounts of filter paper which was placed in such a way as to allow the same amount in each flask to protrude above the surface of the liquid. They were inoculated, as shown in the table, with equal amounts of suspensions of *T. kőningi* spores and of *A. chroococcum* grown previously on Ashby's agar. After six days' incubation at room temperature the pH was measured and the cellulose determined

by the Kiesel and Semiganowsky method (1927). This method depends upon the elimination of all other carbohydrates and the hydrolysis of the cellulose to glucose which is determined by any convenient method. I used the Bertrand (Hawk, 1923) or the Stiles Peterson and Fred (1926) method. Duplicate determinations checked to within 0.2 cc. of the standard solution used. In table 1 the amount of cellulose is not shown, but instead the number of cubic centimeters of standard  $\text{KMnO}_4$  solution used in Bertrand's method of sugar determination.

Table 1 shows that a very significant lowering of the pH in media containing  $(\text{NH}_4)_2\text{SO}_4$  can be brought about without a measurable amount of cellulose decomposition. Furthermore, it shows that the "physiological efficiency" of an organism as measured by Sanborn is probably its nitrogen uptake. These results are different from Sanborn's in that *A. chroococcum* had little or no effect on the change of pH. To explain this discrepancy, we must remember that *T. köningi*, as shown by Heukelelian and Waksman (1925), decomposes cellulose completely to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ , leaving little or no intermediate by-products. Now if we had a cellulose decomposing organism which formed by-products utilizable by *A. chroococcum*, we ought to get a development of *A. chroococcum* which would utilize the nitrate or ammonium radical, and therefore we should get a quicker change to the basic or acid reaction. This might happen without any difference in the amount of cellulose decomposed. To test this possibility, experiments like the former, but with the China blue-rosolic acid indicator (Bronfenbrenner, 1918) recommended by Sanborn, were set up. The medium to contain the ammonium salt was adjusted to a reaction bright red to the indicator, and the medium to contain the nitrate to a distinct blue. They were inoculated as shown in tables 2 and 3. The experiment was repeated, with similar results.

Except for the *T. köningi* cultures, these results are exactly like Sanborn's, so far as the ammonium sulphate cultures are concerned. The results with potassium nitrate are perfectly compatible with his if we admit that the change in reaction is not due to the organic acids of cellulose decomposition but to selective

TABLE 2

*The effect of Azotobacter chroococcum on change in reaction in the decomposition of cellulose by Spirochaeta cytophaga, Trichoderma kőningi, "Mycelia sterila," and "Y" (Dubos) in media containing ammonium sulphate and China blue-rosolic acid indicator (6 days' incubation)*

FLASK NUMBER	INOCULATION	COLOR OF SOLUTION	REACTION
1	Control	Red	Basic
2	Spirochaeta	Less red, turning blue	Slightly acid
3	Spirochaeta and Azotobacter	Bright blue	Distinctly acid
4	Mycelia sterila	Less red, turning blue	Slightly acid
5	Mycelia sterila and Azotobacter	Blue	Distinctly acid
6	Strain "Y" (Dubos)	Less red, turning blue	Slightly acid
7	"Y" and Azotobacter	Blue	Distinctly acid
8	Trichoderma	Blue	Distinctly acid*
9	Trichoderma and Azotobacter	Blue	Distinctly acid*

\* Cultures 8 and 9 changed in reaction at approximately the same time and rate.

TABLE 3

*The influence of Azotobacter chroococcum on change in reaction in the decomposition of cellulose by microorganisms in media containing potassium nitrate and China blue-rosolic acid indicator (9 days' incubation)*

FLASK NUMBER	INOCULATION	COLOR OF SOLUTION	REACTION
1	Control	Blue	Acid
2	Spirochaeta	Violet	Less acid
3	Spirochaeta and Azotobacter	Red	Basic
4	Mycelia sterila	Violet	Less acid
5	Mycelia sterila and Azotobacter	Red	Basic
6	Strain "Y" (Dubos)	Violet	Less acid
7	"Y" and Azotobacter	Red	Basic
8	Trichoderma	Red	Basic*
9	Trichoderma and Azotobacter	Red	Basic*

\* Cultures 8 and 9 changed in reaction at approximately the same time and rate. There was, however, a slight reddish tint on the paper of flask 9 before it appeared in flask 8. This "base," however, was never sufficient to make the solution different in color from flask 9. Thus it may be true, as Heukelekian and Waksman suggest, that *T. kőningi* forms a very minute amount of intermediate material which, however, may be quickly transformed into CO<sub>2</sub> and H<sub>2</sub>O.

absorption of ions. It was noticed in all cultures containing *Azotobacter* except those also containing *T. köningi* that there was a good development of *Azotobacter* on the exposed paper, detected by the typical slimy growth turning dark. These cultures also changed reaction more quickly and the change was first evident on the paper where the growth of *Azotobacter* was seen. My explanation is that the by-products of the cellulose decomposition were utilized by *A. chroococcum* which used the ammonium or the nitrate radical as a source of N, hastening the change in reaction. It is known that the by-products of *S. cytophaga* support growth of *A. chroococcum* (Hutchinson and Clayton,

TABLE 4

*The influence of the growth of Azotobacter on the decomposition of cellulose by Spirochaeta cytophaga (5 months' incubation)*

FLASK NUMBER	INOCULATION	SOURCE OF NITROGEN	CELLULOSE DECOMPOSED
			per cent
1	Control	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	
2	Spirochaeta	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	46
3	Spirochaeta	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	50
4	Spirochaeta and Azotobacter	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	50
5	Spirochaeta and Azotobacter	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	49
6	Control	KNO <sub>3</sub>	
7	Spirochaeta	KNO <sub>3</sub>	49
8	Spirochaeta	KNO <sub>3</sub>	48
9	Spirochaeta and Azotobacter	KNO <sub>3</sub>	46
10	Spirochaeta and Azotobacter	KNO <sub>3</sub>	46

1913). But *T. köningi*, which is known to produce few or no intermediate products, cannot support *A. chroococcum*, and there is no change in reaction.

Next, it was desirable to know if this more rapid change in reaction with cultures containing *A. chroococcum* was accompanied by more complete decomposition of cellulose, that is whether their "physiological efficiency" was increased. The medium changes so rapidly towards a reaction toxic to *A. chroococcum* whenever an ammonium salt is present that it was feared that nothing could be learned unless this was prevented. Accordingly, an excess of CaCO<sub>3</sub> was added to the medium which was otherwise



unadjusted. Cultures of *S. cytophaga* and *A. chroococcum* were inoculated as before, only in this case the paper was moved about aseptically at intervals so as to equalize more nearly the chance for decomposition. When it was converted into a slimy mass it was pushed to the bottom of the flasks and allowed to incubate for five months. At the end of this time the cellulose was determined and the pH measured, as shown in table 4.

Although there was a variation between cultures, it is evident that *A. chroococcum* did not influence markedly the amount of cellulose decomposed by *S. cytophaga*. Similar results with the sterile mycelium are shown in table 5. Here, the paper was not

TABLE 5

*The effect of the growth of Azotobacter on the decomposition of cellulose by Mycelia sterila (6 months' incubation)*

FLASK NUMBER	INOCULATION	SOURCE OF NITROGEN	pH	CELLULOSE DECOMPOSED  per cent
1	Control	KNO <sub>3</sub>	6.92	
2	Mycelia	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	4.85	65
3	Mycelia	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	4.17	49
4	Mycelia and Azotobacter	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	4.06	60
5	Mycelia and Azotobacter	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	4.33	49
6	Mycelia	KNO <sub>3</sub>	7.68	43
7	Mycelia	KNO <sub>3</sub>	7.51	65
8	Mycelia and Azotobacter	KNO <sub>3</sub>	8.27	60
9	Mycelia and Azotobacter	KNO <sub>3</sub>	8.05	47

touched after once being placed in the flask and, since exactly equal amounts could not be placed above the surface of the liquid, the only location where decomposition takes place rapidly with molds, the differences between replicates were large. The reaction was about pH 7; CaCO<sub>3</sub> was not added. Table 5 shows the results with the *Mycelia sterila* culture.

Due to the conditions necessary for growing molds in liquid culture with filter paper, the results are not as striking as with *S. cytophaga*. However, they indicate that no great increase in cellulose-decomposing ability, if any, is effected by *A. chroococcum* when grown with the mold, even though tables 2 and 3 show that the reaction change was very much modified in the early stages of

growth. The heavy growth of *Azotobacter* in this experiment (as well as the previous ones on the decomposition products of the sterile mycelium) shows that the statement of Waksman (1927) that cellulose is decomposed by molds completely to  $\text{CO}_2$  and  $\text{H}_2\text{O}$  is not universally true. It may, however, be true in most cases.

#### DISCUSSION

Sanborn's results are in nearly every case perfectly compatible with mine. Even though it was the cellulose which was decomposed in his experiments rather than the other substances of the crude cotton, the rapid change in pH in the cultures containing *A. chroococcum* could well have been caused by the withdrawal of the ammonium radical by *Azotobacter* which utilized the intermediate products of the decomposition of the cellulose by the other organism in the culture. It is known that both *S. cytophaga* and the cellulose bacteria of Kellerman and his associates (genus "Cellulomonas" like Sanborn's organism) do not decompose cellulose completely to  $\text{CO}_2$  and  $\text{H}_2\text{O}$  but elaborate several organic by-products. Thus my explanation should fit in with the known physiology of the organisms. Also, *T. kőningi*, which has been shown by others to secrete few or no intermediate products, is not affected by associative action of *A. chroococcum*. Furthermore, *A. chroococcum* is known to utilize a number of organic compounds as sources of energy, including products of cellulose decomposition, and is also known to use ammonium and nitrate salts in preference to atmospheric nitrogen. These two facts also fit in with the explanation I have given. These points may be verified by reference to original papers cited by Waksman (1927) and Bonazzi (1921).

#### SUMMARY

1. In media containing an ammonium salt and cellulose, *Azotobacter chroococcum* increased the rapidity of change toward an acid reaction in the decomposition of cellulose by *Spirochaeta cytophaga*, a strain of sterile mycelium, and the "Y" organism of Dubos.

2. The same effect on the rapidity of change toward a basic reaction was evinced in a medium containing  $\text{KNO}_3$ .

3. *A. chroococcum* had little or no effect on the change of reaction produced by *Trichoderma kőningi*.

4. The increased change in reaction was very probably due to the utilization by *A. chroococcum* of the by-products formed in cellulose decomposition. *A. chroococcum* was thus able to develop by using the inorganic nitrogen, leaving the medium more acid in presence of  $(\text{NH}_4)_2\text{SO}_4$ , and more basic when the nitrate was used. Cellulose cultures containing *T. kőningi*, which is known to form few or no intermediate products in cellulose decomposition, was therefore unaffected by the presence of *A. chroococcum*.

5. *A. chroococcum* had no noticeable effect on the amount of cellulose decomposed by *T. kőningi* or *S. cytophaga*.

6. It is not necessary to postulate the existence of a growth-promoting factor secreted by *A. chroococcum* to explain the change in hydrogen ion concentration in cellulose-decomposing cultures containing this organism.

7. Sanborn's data may be explained in a similar way.

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# THE UTILIZATION OF NITROGENOUS ORGANIC COMPOUNDS AND SODIUM SALTS OF ORGANIC ACIDS BY CERTAIN SOIL ALGAE IN DARKNESS AND IN THE LIGHT

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Bacteriologists are not concerned alone with bacteria, but with other microorganisms as well. Files of the JOURNAL OF BACTERIOLOGY show that protozoa, yeasts, molds and viruses have attracted the attention of many bacteriologists, and that this tendency to study fields of microbiology other than bacteriology itself has persisted ever since the early days of our science, when Pasteur occupied himself with any "microbe" that happened to engage his attention. There is a field of microbiology, namely phycology, which, however, has been all but ignored by bacteriologists. There is no good reason why the study of unicellular algae is not a part of the task of the bacteriologist. Morphologically, the lack of a discrete nucleus and reproduction by transverse fission link the bacteria and blue-green algae, and physiologically many green and blue-green algae and bacteria have more in common than do bacteria and protozoa. The technique of isolation and pure-culture study of algae is a technique known in its essential points to bacteriologists and for the most part foreign to the majority of botanists.

The importance of unicellular and microscopic filamentous algae is probably very great. Their value as a food for fish and other water animals has often been stressed. In the soil, where they sometimes exist in hundreds of thousands per gram, (Bristol-Roach, 1923, 1928b), they can, as autotrophic plants, add to the store of soil organic matter and build up compounds which may

serve as a source of energy for nitrogen-fixing bacteria. The symbiosis between algae and *Azotobacter* has been demonstrated repeatedly (Nakano, 1917). There is no evidence that green algae alone fix nitrogen and all experiments which would tend to demonstrate such fixation have been with impure cultures containing possibly nitrogen fixing bacteria, or else the chemical technique has been faulty (Schramm, 1914, Bristol and Page, 1923). Recently Drewes (1928) has demonstrated conclusively the fixation of nitrogen by certain blue-green forms in pure cultures. Algae, also, are known to compete with higher plants for the soil's available nitrogen (Bristol, 1923). In rice culture, they are essential for keeping the  $O_2$  concentration high, and from the work of Wakabayashi (1924) one would suppose that they would be of advantage in cranberry bogs. As heterotrophs, algae also live in a non-resting condition in the deeper layers of the soil (Bristol-Roach, 1927) where they can act as do the bacteria and molds in the decomposition of organic matter. For a summary of our knowledge of the soil algae, the reader is referred to Bristol-Roach (1923, 1928) and Waksman (1927).

#### HISTORICAL

It was a leading bacteriologist, Beijerinck (1889), who first succeeded in obtaining algae in pure culture, free not only from other species of algae, but from bacteria as well. A gelatin medium was used and the colonies were picked and purified in a way similar to that in which bacteria are usually handled. He isolated two species, one of which caused a liquefaction of the gelatin. Since that time there have been many studies of pure cultures of algae. The important work of Chodat and associates was summarized in a monograph (Chodat, 1913). This monograph and a paper by Nakano (1917) give a good summary of the work done up to 1917. Since then, there have been many notable studies, several of which are reviewed in a paper by Topali (1923). The technique of isolation is given by Chodat (1909) and Schramm (1914). One can find the method of isolation from soil, a somewhat more difficult task than isolation from other habitats, in Bristol-Roach's treatment in Abderhalden's Handbuch (Waksman et al., 1927).

That algae do not necessarily obtain their energy exclusively by photosynthesis, but that some of them can also live saprophytically in complete darkness like bacteria and yeasts, is a discovery sometimes credited to Artari (1899). Previous to this, however, Klebs (1887) had announced this finding, and Bouilhac (1898) had found that a species of *Nostoc*, a blue-green form, grew and produced chlorophyll in complete darkness in a mineral salt solution containing glucose. Whether the algae grew at the expense of the glucose or the bacterial contaminants, Bouilhac could not say, but the fact that the chlorophyll was produced in the absence of light was proved by analysis of the spectral bands of the culture. Beijerinck (1898) grew pure cultures of green algae in absolute darkness for several years on maltwort gelatin and noted the production of green pigment. Artari (1899), independently of Beijerinck, grew certain algae in pure culture in darkness and found that the green pigment was produced and Radais (1900) very shortly afterwards confirmed this observation and, in addition, proved by spectroscopic methods that the green pigment produced by his pure cultures was actually chlorophyll. It is only fair to remark that both Radais and Artari gave credit to previous workers. These findings have been confirmed many times. One investigator (Dangeard, 1921) cultivated his algae for eight years by successive plantings, and, at the end of this time, there was no difference in the amount of green pigment produced by cultures incubated in darkness and those which had been incubated in the light for that length of time.

The fact that some of the algae can live either autotrophically by photosynthesis, or heterotrophically in darkness at the expense of sugar or other proper organic nutrient, while producing chlorophyll at the same time, makes the algae of extreme interest to biologists. We are justified in making an absolute denial of the statement, so often encountered, that light is necessary for chlorophyll formation. Likewise, material is thus available for controlled work on the production of chlorophyll and the physiology of photosynthesis. Emerson (1929a, b) was able to use algae to great advantage in this sort of work. Also, we have here a group of plants midway between the saprophytes, such as fungi and



bacteria, and the higher green plants, connecting them physiologically as well as morphologically. That so many plant physiologists have neglected, except for purely nutritional work, pure culture study of unicellular algae is hard to explain. The technique is not much more difficult, only more time-consuming, than ordinary bacteriological procedures.

As to the nutrition of the algae, it may be stated that one species varies from another as much as do bacteria. Obviously any studies on the nutrition of algae except in pure cultures are of little value, and such studies will be ignored here for the most part. The statements of many authors that their cultures were pure cannot be relied upon in all cases, for very often what is meant is that only one species of alga was present, bacterial contaminants being ignored. One can in nearly all cases tell from the context whether their cultures were really "absolutely pure" or merely "species pure." The isolations of algae have been of the unicellular or small filamentous species, since the difficulty of isolating larger forms increases enormously with the size of the plant. Few isolations of blue-green species have been made, due to technical difficulties. Some of the isolated forms have been obligate autotrophs and will not grow in absence of light. Others grow poorly in absence of light; others grow in darkness on proper organic media, but produce no chlorophyll; others produce chlorophyll; still others grow much better heterotrophically and make no use of sunlight if a proper concentration of sugars is present.

The utilization of various carbohydrates has been studied by many workers. Glucose has almost invariably been found to be most available to algae, but there is a great difference in the nutritional value of many of the other carbohydrates. The usual hexoses, pentoses, disaccharides and higher alcohols used in bacteriological procedure have been used in classifying and studying algae, notably by Artari (1909, 1913), Chodat (1913), Nakano (1917), and Bristol-Roach (1926, 1927).

Nitrogenous organic compounds have been studied extensively for their nitrogen availability to algae, but very little as a source of carbon. Beijerinck (1889) noted a liquefaction of gelatin by one

of his species, and liquefying power is one of the criteria used in classifying microscopic algae, notably by Chodat (1913) and Nakano (1917). Peptone has also been used, but usually in the presence of glucose, or else in the sunlight. Thus it is hard to say, with most of the studies of algae growing in peptone media, whether the peptone was acting as a superior nitrogen source or also as a source of carbon. Various investigators have grown algae in total darkness, with gelatin as the sole source of energy. All species of algae so far studied in pure culture by no means utilize gelatin or peptone, but it is established that some of them do. Artari (1913) found that asparagin may serve both in the capacity of nitrogenous and carbonaceous food, as did also alpha-alanin, acetamide and glyocol. None of these substances were nearly as good as glucose, however. Pringsheim and Mainx (1926) have shown that certain algae grow better in the presence of wort or meat extract, of peptone, gelatin or casein digested with trypsin, and of amino compounds, especially asparagin and glyocol, than in their absence.

Henri Tanner (1923) made an intensive study of the liquefaction of gelatin by the cultures of the genus *Scenedesmus*, contained in Chodat's extensive museum. Since gelatin incorporated in a mineral salt medium was liquefied very slowly indeed, but much more rapidly when glucose was also present, it would appear that the gelatin was acting as a source of nitrogen rather than of carbon. Certain species also grew in sterilized milk, although lactose was unavailable to the species studied. A clearing of a zone around the colonies of algae growing on casein-mineral salt agar showed the production of exo-proteases. The para-cresol-tyrosinase reaction showed the further digestion of rennit-digested milk, and of edestin. Usually the proteins were digested only to peptones, although in some cases amino-acids were also formed. Tanner thought that the algae used these proteins saprophytically but, although this may have been correct, it is not certain that these compounds were not acting as a nitrogen source in most of the cases. Had his cultures been incubated in the absence of light there would have been no question as to the ability of the substances to serve as the sole source of energy to the algae.

There is also little definite work on the exclusive utilization of organic acids or their salts by algae. Bokorney's (1918) work was most likely with impure cultures. It is difficult to conceive that he had purified his culture of *Spirogyra* and he made no definite claim that he had. The same criticism of impure cultures applies also to Treboux's (1905) work, as well as to that of Ternetz (1910). The former found that calcium acetate in particular, and also in some cases, calcium lactate, citrate and butyrate supported growth of algae in darkness. Due to the great doubt as to the purity of the culture, this study is of only limited value, for the nutrients supplied the algae could well have come from the decomposition products of the compounds, or from the products of autolyzed bacterial cells. The control of the pH also would seem to be imperative, due to the rather great alkalinity of some of the salts of acids ionizing so poorly. Naturally, these early workers did not consider this sufficiently. The only definite results on organic acids as a source of energy for algae grown in darkness in the absence of other sources of carbon noted by the writers in the literature were those of Chodat (1913), Mendrecka (1913), and Artari (1913). The latter found ammonium tartrate to produce slight but definite growth of *Chlamydomonas Ehrenbergii*. Chodat found that in darkness *Cystococcus Cladoniae-pyxillatae* was able to grow slightly on prolonged incubation on agar media to which had been added potassium tartrate and Mendrecka found the same to be true of *Chlorella variegata* on potassium acetate and citrate agar. Letellier (1917), also working with Chodat, found that potassium oxalate and citrate in Detmer's (1/3) agar reduced the growth of three species of *Cystococcus* when grown in the light, and failed to support growth in darkness.

The present study was undertaken to find out more definitely the possibility of the utilization of certain nitrogenous organic compounds and organic acids by soil algae, as the sole source of carbon and energy, that is, when grown heterotrophically in total darkness, and to determine their effect as added nutrients on the growth of algae in the light.

## EXPERIMENTAL

The following cultures of algae were used :

- A. *Chlorella*, *sp.* isolated from Minnesota soil.
- B. *Pleurococcus*, *sp.* isolated from soil by Bristol-Roach.<sup>1</sup>
- C. *Cystococcus*, *sp.* isolated from soil by Bristol-Roach.<sup>1</sup>
- D. *Scenedesmus costulatus* var. *chlorelloides*, isolated from Minnesota soil.
- E. *Unidentified species* isolated from Minnesota soil.

Strains A, D, and E are not identified with certainty as yet; hence, they will be kept in culture until accurate identification is possible.

The basic nutrient solution used is that of Beijerinck as modified by Moore (Waksman, 1927) :

NH <sub>4</sub> NO <sub>3</sub> .....	0 5 gram
KH <sub>2</sub> PO <sub>4</sub> .....	0 2 gram
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0 2 gram
CaCl <sub>2</sub> .....	0 1 gram
FeSO <sub>4</sub> .....	Trace
H <sub>2</sub> O (distilled).....	1000 cc.

The organic acids and the above chemicals used were of the C.P. grade; the casein was "nach Hammersten," the egg albumin, Merck's impalpable powder, and the peptone, glucose, and gelatin, Difco brand. The agar-agar used (15 gram per liter) was also Difco brand. The organic acids and nitrogenous compounds were dissolved in a little water and brought to about pH 6.0 with NaOH before being added to the rest of the media. They were used at the rate of 5 grams per liter of the finished medium, which was again adjusted to pH 6.0 with NaOH or HCl just before sterilization (twenty minutes at 121°C.)

<sup>1</sup> These cultures were kindly supplied by Dr. Bristol-Roach to the senior author in 1926. She also was kind enough to demonstrate the technique of isolation of pure cultures of algae from soils. Most of all, he is indebted to her for her insistence on the necessity of accumulating physiological data on soil algae before drawing conclusions as to their activities or importance in the soil itself. The purity of all cultures was established by repeated microscopic examination of stained and unstained preparations which failed to reveal any bacteria, as well as by the lack of growth after inoculation on several different bacteriological media.

For the first experiment five large agar slants each containing 20 cc. of agar were prepared from each of the following agents; plain (no organic food added), glucose, peptone, casein, gelatin, albumin and lactic, succinic, malic, oxalic, tartaric, and citric acids. These were inoculated with each of the 5 species of algae. Three tubes of each were placed in boxes, which were covered, wrapped in dark paper and placed in a dark chamber, and two in tumblers on the window-sill of a room on the north side of a building. They were incubated for forty-two days and readings

TABLE 1

*The effect of various organic compounds on the growth of algae on Moore's solution agar, pH 6.0 in darkness (42 days' incubation)\**

MEDIA	PLEURO-COC- CUS SP.	CYSTOCOC- CUS SP.	CHLO- RELLA SP. (?)	SCENE- DESMUS COSTULA- TUS VAR. CHLOREL- LOIDES (?)	UNIDEN- TIFIED SPECIES
1. Plain.....	+	+	+	+	+
2. Albumin.....	++	++	+++	+++	±
3. Casein.....	++	++	+++	++	++
4. Gelatin.....	++	++	++±	++	±
5. Peptone.....	+++	++	+++	++	+++
6. Citric.....	+	+	+++		±
7. Lactic.....	+	++	++	±	++
8. Malic.....	+	±	±		+
9. Succinic.....	+	+	±	++	±
10. Tartaric.....	+	++	++	±	++

\* Each of the authors recorded the growth separately on two successive days. The cultures which did not agree in all four recordings are marked ±. +, ++, +++ = degrees of growth.

made. The results shown in table 1 are only for those grown in darkness. All tubes grew in the light, although the oxalate medium showed less growth than the control, due possibly to the precipitation of the calcium (Pringsheim, 1926). It is evident that none of the compounds except possibly the oxalate was toxic.

The results are somewhat confusing. The control tubes of all 5 species grew in the darkness. This on first thought might be explained in one of three ways. First, the light may not have been entirely excluded. This was not the case, however, since

cultures had been grown previously in the same place without the slightest growth. Cultures grown in liquid media, the results of which are shown later, were also entirely negative. The very definite growth was evidently not due to light, which was entirely excluded. Secondly, the organisms might have utilized the agar itself as a source of energy. Chodat (1913) explained the very slight growth of certain algae grown on inorganic nutrient agar media in the darkness as due to such utilization. While this is possible, some other reason must probably be sought. The growth was due in all likelihood to hydrolytic products of the agar. Sterilization of the acid medium used, or of the medium of Chodat (Detmer 1/3), would be expected to hydrolyze the agar slightly, but sufficiently to allow a slight growth of some species. Then too, agar even of the best grade is difficult to procure free from organic nitrogen (Fellers, 1916a, b). Agar-agar is not a carbohydrate proper, but an organic ester combined with bases, principally Ca, and often with small amounts of nitrogenous compounds (Hoffman and Gortner, 1925). This explains why it is impossible to wash the Ca or the nitrogen completely out of the agar. Thus, even if a neutral medium is sterilized without hydrolyzing the agar, the impurities are sufficient to allow a slight growth of many organisms (Fellers, 1916a). We have found that several species of algae grown on neutral inorganic agar media produced a very slight growth on prolonged incubation in absolute darkness, whereas no development was ever evident in inorganic liquid media. However, the growth was not nearly so pronounced as the growth shown in the above cultures. Therefore, it was concluded that these algae grew principally at the expense of the agar hydrolytic products.

The results in table 1, therefore, do not show whether or not any of the organic materials can serve as a sole source of energy. They do show, however, that several of them cause an amount of growth greater than that provided by the meager nutrient material in the hydrolyzed agar.

To show whether or not the materials would serve as a *sole* source of energy, it was thought advisable to use liquid media. Tubes containing 30 cc. were used and incubated as before, except

that the tubes in the light were placed on a shelf in the laboratory, since it was feared that the severe cold of a Minnesota winter would be harmful if the tubes were placed on the window-sill. They were inoculated with a loopful of a suspension of the algae grown on inorganic agar thoroughly mixed in a physiological

TABLE 2

*The effect of organic compounds on the growth of Scenedesmus costulatus var. chlorelloides (?) grown in diffuse light in Moore's solution*

MEDIA	75 DAYS' INCUBATION		89 DAYS' INCUBATION	
	Number of cells counted	Number of cells per cubic centimeter (thousands)	Number of cells counted	Number of cell per cubic centimeter (thousands)
Plain.....	149	118	126 127	139 141
Albumin .....	831	576	1,034 1,045	1,148 1,160
Casein.....	173	192	192 205	213 234
Gelatin.....	357 185	219 205	451 478	501 531
Peptone.....	Very heavy growth, much heavier than glucose; counts lost		1,045 767	10,500 6,730
Lactic.....	249	276		
Malic.....	248	137	706 535	784 592
Succinic.....	279	258	502 545	557 605
Glucose.....	594	549		

salt solution. This light inoculation, was sufficient. In about seventy-five and ninety days the cultures were examined and, in order to get a more definite idea of the amount of growth, the algae were counted by means of a counting chamber. They were vigorously shaken before counting. The results are shown for

TABLE 3

*The effect of organic compounds on the growth of Chlorella sp. (?) grown in diffuse light in Moore's solution*

MEDIA	74 DAYS' INCUBATION		93 DAYS' INCUBATION	
	Number of cells counted	Number of cells per cubic centimeter (thousands)	Number of cells counted	Number of cells per cubic centimeter (thousands)
Plain.....	94	104	217	241
Albumin.....	287	1,645	302	3,020
Casein.....	200	222	310	344
Gelatin.....	220	244	441	489
			376	417
Peptone.....	371	3,760	661	6,610
			702	7,020
Malic.....	490	544	864	959
Succinic.....	415	461	643	714
			687	763
Glucose.....	459	849	331	367
			361	668

TABLE 4

*The effect of organic materials on the growth of Cystococcus sp., Pleurococcus sp., and unidentified alga grown in diffuse light in Moore's solution*

MEDIA	CYSTOCOCCUS SP. (93 DAYS' INCUBATION)	PLEUROCOCCUS SP (85 DAYS' INCUBATION)	UNIDENTIFIED SPECIES (87 DAYS' INCUBATION)
Plain.....	+	+ 56,105 per cc.	+ 88,000 per cc.
Albumin.....	++	+	+
Casein.....	+	+	+++ 62,880,000 per cc.
Gelatin.....	+	+	+
Peptone.....	+	++ 5,700,000 per cc.	+
Citric.....	+	+	+
Lactic.....	+	+	+
Malic.....	+	+	+
Succinic.....	+	+	+
Tartaric.....	+	+	+
Glucose.....	++++	+++ 68,000,000 per cc.	+++ 10,400,000 per cc.



the cultures grown in the light in tables 2, 3 and 4. In some cases counts of duplicate tubes are given in order to show the variability, and, in most cases, the actual number of cells counted, in order to show the reliability.

These tables show that in the presence of many organic com-

TABLE 5

*The effect of organic materials on the growth of algae in total darkness. (Control contained no cells)*

MEDIA	NUMBER OF CELLS COUNTED	NUMBER OF CELLS PER CUBIC CENTIMETER (THOUSANDS)
<i>Scenedesmus costulatus</i> (85 days' growth)		
Albumin.....	691	383
Casein.....	78	43
Peptone.....	55	30
Glucose.....	200	138
<i>Chlorella</i> sp. (89 day's growth)		
Albumin.....	457	253
Casein.....	68	37
Glucose.....	145	80
<i>Pleurococcus</i> sp. (92 day's growth)		
Casein.....	1,159	2,318
Glucose.....	802	200,500
<i>Cystococcus</i> sp. (94 days' growth)		
Glucose.....	1,150	638
<i>Unidentified species</i> (88 days' growth)		
Casein.....	41	22
Glucose.....	395	98,750

pounds, both nitrogenous and non-nitrogenous, a considerably greater growth of algae results in diffuse sunlight than in its absence. To know whether or not these compounds served as a sole source of carbon and energy, counts were made of the cultures grown in the darkness. Any tube showing the slightest green growth was counted. Several examinations of the control inor-

ganic tubes failed to reveal any alga cells at all. The results are shown in table 5.

TABLE 6

*The effect of organic compounds on the growth of Scenedemus costulatus var. chlorelloides in Moore's solution in total darkness—Bristol-Roach's dilution method (Waksman et al., 1927) (128 days' incubation)*

ORGANIC COMPOUND	NUMBER POSITIVE TUBES FROM EACH DILUTION													MOST PROBABLE NUMBER OF VIABLE UNITS PER CUBIC CENTIMETER
	5:40	5 80	5 160	5:320	5:640	5:1,280	5 2,560	5:5,120	5:10,240	5 20,480	5 40,960	5 81,920	5:163,840	
None.....	0	0	0	0	0	0	0	0	0	0	0	0	0	18,751
Casein.....	3	3	3	3	3	3	3	3	3	3	3	2	1	
Gelatin.....	3	3	3	3	3	3	3	3	3	2	2	1	0	5,938
Citric acid.....	3	3	3	3	3	3	3	3	3	0	0	0	0	1,331
Lactic acid.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Malic acid.....	3	3	3	3	3	3	3	3	3	3	2	0	0	5,938
Succinic acid.....	3	3	3	3	3	3	3	3	3	3	0	1	0	4,534
Tartaric acid.....	3	3	3	3	3	3	3	3	3	2	1	0	0	3,543
Oxalic acid.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0

TABLE 7

*The effect of organic compounds on the growth of Chlorella sp. in Moore's solution in total darkness (5 tubes of each dilution inoculated) (150 days' incubation)*

ORGANIC COMPOUND	NUMBER POSITIVE TUBES PER DILUTION				MOST PROBABLE NUMBER OF VIABLE UNITS PER CUBIC CENTIMETER*
	1:100	1:1,000	1:10,000	1:100,000	
None.....	0	0	0		(Less than 20)
Albumin.....		5	5	5	180,000+
Peptone.....		5	5	4	160,000
Citric acid.....	0	0	0		
Malic acid.....		5	4	2	25,000
Lactic acid.....	0	0	0		
Tartaric acid.....		5	1	0	3,500
Oxalic acid.....	0	0	0		
Succinic acid.....		5	4	0	13,000

\* For different mathematical interpretation of these data and those of tables 8 and 9, the formula and tables of Halvorson, soon to be published, may be consulted. These "numbers" are minimum figures and are smaller than in the former tables, since clumps are counted as single organisms. In contrast to the counting-chamber method, only viable cells are counted.

The lack of development in media containing organic acids is striking; but it can be seen that peptone, casein, and egg albumin,

as well as glucose, supported the growth of several species of algae. However, these substances are not equally valuable, as some

TABLE 8

*The effect of organic compounds on the growth of Cystococcus sp. in Moore's solution in total darkness (5 tubes of each dilution inoculated) (178 days' incubation)*

ORGANIC COMPOUND	NUMBER OF POSITIVE TUBES PER DILUTION				MOST PROBABLE NUMBER OF VIABLE UNITS PER CUBIC CENTIMETER
	1:100	1:1,000	1:10,000	1:100,000	
None .....	0	0	0		
Albumin .....		5	2	0	5,000
Casein .....	5	1	0		350
Casein duplicate .....	5	1	0		350
Peptone .....		5	1	0	3,500
Citric acid .....	5	1	0		350
Lactic acid .....		5	3	0	8,000
Malic acid .....	0	0	0		
Oxalic acid .....	0	0	0		
Succinic acid .....		5	3	1	11,000
Tartaric acid .....		5	3	0	8,000

TABLE 9

*The effect of organic compounds on the growth of Pleurococcus sp. and unidentified species grown in Moore's solution in total darkness (5 tubes of each dilution inoculated)*

ORGANIC COMPOUNDS	NUMBER OF POSITIVE TUBES PER DILUTION				MOST PROBABLE NUMBER OF VIABLE UNITS PER CUBIC CENTIMETER
	1:100	1:1,000	1:10,000	1:100,000	
<i>Pleurococcus</i> sp. (195 days' incubation)					
None.....	0	0	0		
Casein.....		5	4	4	35,000
Albumin.....		5	5	1	35,000
Peptone, gelatin (3 replicates) and all the acids, negative in all dilutions					
<i>Unidentified species</i> (193 days' incubation)					
None.....	0	0	0		
Albumin .....		5	5	0	25,000
Peptone, gelatin, and all the acids, negative in all dilutions					

species failed to develop on some of the nitrogenous compounds. The remaining tubes were filled with sterile distilled water

to the 30 cc. mark to make up for the water which had evaporated, and were reincubated in the same dark cupboard, again being carefully wrapped. They were not allowed in the light more than the time necessary for shaking and taking the sample.

After some weeks the algae were again counted, this time by means of the dilution method, in order to detect growth which was too meager to find by the counting chamber method. This method, unfortunately not as widely used in bacteriology as it should be, consists in making a series of dilutions, e.g., 1:100, 1:1000, 1:10,000, etc., and taking a definite amount from each dilution in several replicates and placing it in favorable media. If enough replicates are used the results are fully as accurate as those of the plate method, and the method is suitable for organisms which do not form a discrete colony or are easily overgrown by other organisms. As in the plate method, clumps are counted as single individuals. In this case peptone, 0.2 per cent—glucose, 1.0 per cent—Moore solution was used. After sufficient time for growth had elapsed the tubes showing growth were counted, and from a table of probabilities the most probable numbers of cells per cubic centimeter were estimated. The formula and table of Fisher (Waksman et al., 1927) and those of McCrady (1918) were used. Dr. H. O. Halvorson has worked out another formula thought to be more accurate for such data, and will publish it with tables in the near future. The method using Dr. Fisher's tables is given by Bristol-Roach (Waksman et al., 1927) in detail. A separate pipette was used for each manipulation. (See tables 6, 7, 8 and 9.)

#### DISCUSSION

These results show that the organic acids were able to support growth of some algae, although it should be noted that very long incubation seemed to be necessary, and that the number of cells even then was relatively small. It is quite possible that still more prolonged incubation would result in greater development. It is also worth noticing that there was a great variation in availability of organic acids. Oxalic acid was not utilized by any of the algae studied. To be sure, this may have been due to

the precipitation of the calcium; yet it is known that calcium is not necessary to all algae (Pringsheim, 1926). Some algae seemed to be unable to use any of the organic acids at all. It is evident that glucose, which is generally so readily available, also caused a much earlier "staling" of the cultures. Chodat (1913) explains this very logically on the basis of a carbon assimilation more rapid than nitrogen intake. We have confirmed this many times when we noted that a readily available N source like peptone prevented or greatly retarded the loss of green color and the "staling. But the organic acids, which are usually less readily assimilated, allowed for a more protracted growth. This "staling" in glucose solutions with decrease in viable cells can be noted from the tables given here.

The organic nitrogen compounds likewise were available to certain algae in varying degrees. The authors believe that it is shown here for the first time that casein and egg albumin may be utilized as the sole source of energy by green algae. It should be noted that merely because one "protein" is available to a certain species or strain of algae, another "protein" may not be utilized at all. This brings up the possibility of identifying specific proteins in a manner comparable to the way in which Castellani (1928) and others identify sugars by various bacteria and *Monilia* forms. The fact that Difco peptone in some cases supported a better but more delayed growth than glucose leads one to suspect that the protein degradation products may prove superior to the proteins themselves as carbon sources for the algae. It is indicated also that compounds which caused a considerable increase in growth when added to inorganic media incubated in the light, or in the darkness in the presence of other organic nutrients, did not necessarily serve as a sole source of energy.<sup>2</sup>

Our experiments cited above, as well as many others performed in this laboratory in the past three years, have convinced us that,

<sup>2</sup>While this paper was in press, Beckwith (Proc. Soc. Exp. Biol. and Med., 28, 1-3, 1929) published a preliminary note on extended physiological research on the genus *Chlorella*. As in our work, it was found that several compounds (carbohydrates, in his studies) which failed to support growth in darkness, caused a considerable increase of growth in light.

for this type of nutritional work, liquid media are superior to agar. The use of electrodialed agar resolidified with pure inorganic bases (Hoffman and Gortner, 1925), or of silica jelly suggests itself at once, and no doubt these solidifying agents will come more and more into use for careful nutrition work in bacteriology. It might be mentioned, however, that for this type of work it is absolutely essential to have a silica gel which can be autoclaved after dialysis. Legg's method (1919) of preparation is suggested.

Finally, the authors wish to express their indebtedness for the help and inspiration which they have obtained from the published work of Professor Chodat and his students. Their work on pure cultures of algae has dominated the field, and in going over the work one is struck by the care and insight with which these studies, prodigious in amount, were carried out. This is all the more remarkable when one considers that the pure culture work on algae is only one of many lines of botanical, bacteriological, and biochemical research carried on by them, as, for example, oxidation enzymes, systematic phycology, cellulose-decomposing bacteria and fungi, the flora of Peru and other exotic localities, plant ecology and probably others unknown to the authors.

#### SUMMARY

1. Pure cultures, free from bacteria, of *Pleurococcus* sp., *Cystococcus* sp., *Chlorella* sp. (?), *Scenedesmus costulatus* var. *chlorelloides* (?) and an unidentified strain of green algae were grown in liquid and agarized Moore's Solution in diffuse sunlight and in total darkness. To these media were added glucose, gelatin, peptone, egg albumin, casein and citric, lactic, malic, oxalic, succinic, and tartaric acids (brought to pH 6.0 with NaOH).

2. The organic compounds (except oxalic acid) caused an increased growth of some of the algae when grown both in light and darkness on slightly hydrolized agar.

3. Glucose and the organic nitrogenous compounds served as the sole source of energy in varying degrees for some of the algae when grown in total darkness in liquid media. The organic acids (except oxalic acid) supported a slight and delayed growth

of three of the species, but none of the algae was able to use all of the acids tested.

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# THE INTERMITTENT GROWTH OF BACTERIAL CULTURES

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In ordinary laboratory experiments bacterial growth is circumscribed by the exhaustion of essential nutrients, or by accumulation of the products of growth. The population is forced by the limitation placed on it by the experimental conditions to increase to the maximum for these conditions and then decline. This does not necessarily show what would take place if the unfavorable factors created by the growth of the bacteria were continuously removed. We can conceive such a condition to exist in the animal body, in which fresh food is constantly supplied, and by-products removed by the blood and other body fluids. Growth in a long tube of fluid medium like that used by Bibb (1925) does not exactly duplicate conditions in the body but permits a fluid colony to extend itself continuously into regions, at most only slightly affected by the previous growth. It is conceivable that nutrients may be drawn by the colony from an area slightly in advance of the growth and that products of growth may diffuse along the tube faster than growth would progress. However, it is not probable that this interchange takes place rapidly enough to affect growth seriously and our experiments indicate that there is no material change in the medium beyond the limits of the colony.

The apparatus used in this work consisted of a pyrex tube 7 mm. in diameter and about 15 meters long, wound in a flat spiral with a flask sealed to the inner end of the tube. The flask was of a capacity to hold sufficient medium to fill the tube completely with a slight excess for inoculation. In autoclaving, the broth

was forced out of the tube but was drawn back as it cooled. All air bubbles were worked out of the tube by careful tilting.

A broth of the following composition was used: Infusion broth, 1000 cc.; pepton, 5 grams; lactose, 0.5 gram.

The small amount of sugar was sufficient to promote a prompt and vigorous growth of *Es. coli* or *Str. lactis* but not enough to produce a change in reaction beyond the normal limits of these organisms. Bromthymol blue was used as an indicator.

The cultures used were fairly active laboratory cultures of *Str. lactis* and *Es. coli*. No essential difference in the growth of these cultures was noted, except that, as would be expected, *Es. coli* grew much more rapidly than *Str. lactis*.

Inoculation was made into the flask, the coil held at 30° and the progress of the growth through the tube marked from time to time by the change in color. Turbidity and decolorization were nearly identical but the demarkation between the decolorized and the unaffected indicator was not always sharp and some irregularity in the results was introduced.

In order to obtain more complete records a motion picture camera was arranged to make a photograph of the coil automatically at fixed intervals.<sup>1</sup>

The small room in which the apparatus was installed was held at 30°. With the culture of *Es. coli* at this temperature the growth covered the entire coil in about eight days. The film obtained in this way was projected to the normal size of the coil and the rate of growth determined. It was sometimes difficult to determine on this reproduction the exact extent of the decolorization but with the *Es. coli* inoculations the reaction of the broth was raised to pH 7.4 and the amount of indicator increased to give a more pronounced change. This reduced the difficulty and while the indefiniteness of the end point produced some raggedness in the curves the results obtained approach very closely the actual conditions.

At first glance there would seem to be no reason why the growth should not proceed at a uniform rate through the entire length

<sup>1</sup> We are indebted to Mr. Harry Greene of the Motion Picture Laboratory for the use of this apparatus.

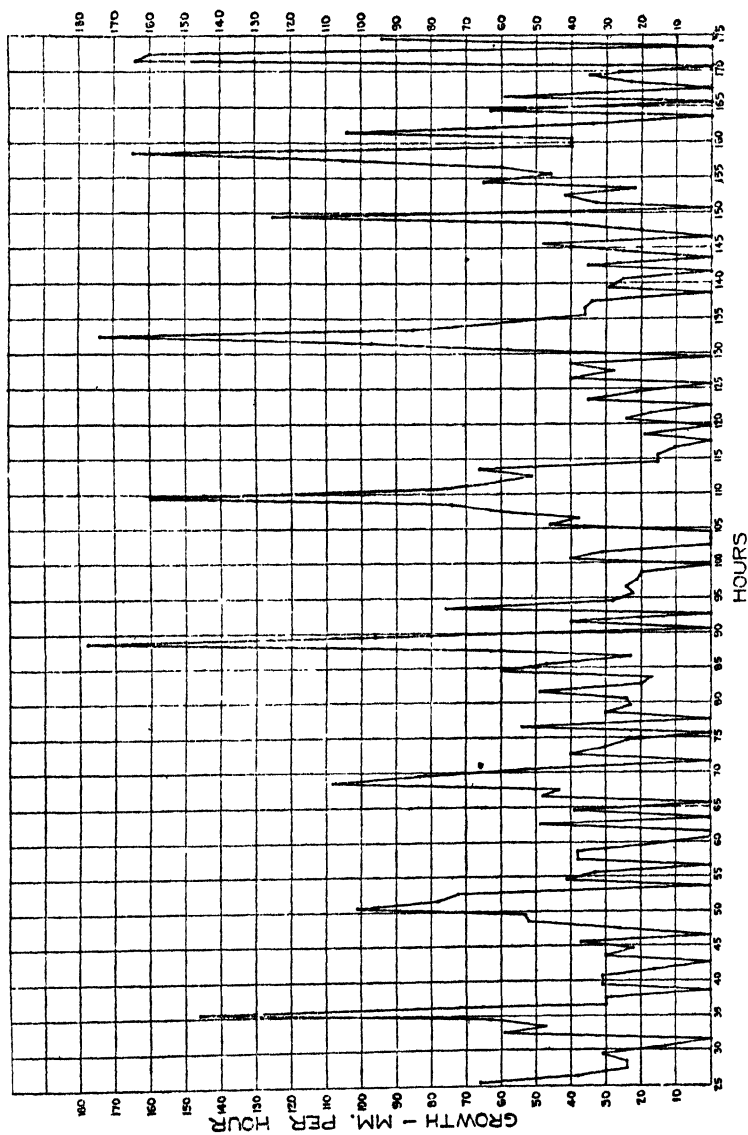
of the tube. Bibb (1925) has reported that this is what occurs. However, we have been unable to confirm his results. The uneven progress of the growth was evident, even on casual observation. Periods of rapid advance of growth were followed by intervals of very slow progress or complete cessation of change.

Figure 1, which is representative of a number of experiments, shows that there is a considerable degree of periodicity to the alternation of rapid and slow growth. In considering this graph it should be kept in mind that the curve represents not extent of growth, but rate of advance of growth for definite time periods. The number of bacteria in the different periods could not be determined.

The objection that variations in rate of growth may have been caused by uneven temperatures, electric currents, or other physical factors connected with the control and the lighting, can be met with observations made on a smaller vertical coil held in an ordinary incubator. Observations could not be made often enough, or for sufficient time, to establish periodic variations, but the marked variation in the rate of growth was very evident.

The growth in the tube is obviously a colony in a liquid medium, pushing its margin out into new territory. The physical conditions under which a colony grows on solid media are so different from those existing in a fluid medium that it would not be safe to reason from one to the other. However, it can be readily demonstrated that the growth of a spreading colony on the surface of an agar plate does not proceed uniformly. Motion pictures with exposures at five-minute intervals were made of spreading colonies and the film projected so that measurements could be made on an enlargement equivalent to  $4\frac{1}{2}$  times the diameter of the colony. Since growth was evidently not taking place uniformly at all parts of the colony, measurements were made along one radius only. These measurements showed marked variations in the rate of growth.

There were frequent intervals of from ten to twenty minutes in which no progress could be detected. One colony which grew 8.4 mm. in one direction in five hours produced practically all of this growth in eighty-five minutes. The growing periods were

FIG. 1. RATE OF GROWTH OF A CULTURE OF *Es. coli* IN A TUBE

distributed fairly uniformly over the entire time. The frequent exposure to an intense light may possibly have been a factor in this alternation of slow and rapid growth.

The rate of growth of a colony on the surface of an agar plate may be influenced by physical conditions which tend to make observations on this type of colony of little value in studying the rate of growth of bacteria. It is a matter of common knowledge that the growth of spreaders is dependent, to some extent at least, on the moisture conditions on the surface of the agar. Bacteriologists who have had persistent trouble with this type of colony in making water and milk counts would be surprised to find how difficult it is to induce a colony to spread for useful purposes. To secure a spreading colony it is usually necessary to use measures which produce a moist surface, as for instance quick cooling of the melted agar.

It is conceivable that differences in the amount of water on different parts of the surface of the agar may account for variations in the rate of growth. This, obviously, would not be comparable with the variations observed in the coil. When plates were made so that the surface was very moist and the growth of a colony of *B. vulgatus* followed under a microscope held at a constant temperature, movement of free cells into the water beyond the limits of the colony could sometimes be observed. When this happened the rapid multiplication of these free cells produced a sudden extension in the limits of the colony. Ordinarily, however, the boundary of the colony was sharply defined and its growth had the appearance of a flowing due to an accumulation of cells in the interior. Growth usually proceeded in the form of streamers with the space between filling in more slowly.

Colonies of *Es. coli* grew continuously but at a rate which varied at times over 100 per cent.

The relation between colony growth and growth in the closed tube seems so doubtful that these data are not included.

Some observations of a different nature may possibly help to explain the variations in the rate of growth in the tube. If a culture of *Str. lactis* is grown in a collodion sac, suspended in a flask of broth containing a very small amount of sugar so that the

reaction will not become unfavorable, as the growth in the sac progresses the broth in the flask becomes more favorable to the initial growth of the same culture. However, when the growth in the sac has reached its maximum the bacterial population which the broth in the flask will maintain has been much reduced. This effect can also be shown by removing portions of a broth culture from time to time and freeing it from bacterial cells by filtration.

The results of an experiment of this nature are shown in table 1. These results are typical of a number of similar experiments made by growing the culture in a collodion sac or by filtration. The broth had the same composition as that used in the coil except that no indicator was used. The results shown in table 1 were obtained by growing *Str. lactis* in a flask held at 30°. At the

TABLE 1

*The accelerating and inhibiting effect obtained in a broth culture of Str. lactis*

AGE  <i>hours</i>	BACTERIA COUNT IN FLASK	TUBE	BACTERIA COUNT IN FILTERED BROTH			
			4 hours	7 hours	11 hours	27 hours
0		A	320,000	2,525,000	40,000,000	710,000,000
4	305,000	B	144,000	1,125,000	11,950,000	665,000,000
8	7,950,000	C	420,000	3,000,000	54,900,000	820,000,000
24	715,000,000	D	8,675,000	8,750,000	8,750,000	7,100,000

time of inoculation, and at four, eight, and twenty-four hours, portions of this culture were filtered into sterile tubes. In each case the flask was inoculated from a fresh milk culture. The bacterial content of the broth at these periods is given in the second column of the table. When the last sample (D) was taken, the normal population for this broth had been reached. The tubes of filtered culture were inoculated with the strain of *Str. lactis* grown in the flask and counts made at four, seven, eleven and twenty-seven hours.

The difference in tubes A and B at four hours are probably not significant but there can be no question about the accelerating effect shown in tube D. This marked effect on the initial growth was always observed in the twenty-four-hour culture and some-

times at an earlier period. At twenty-seven hours, tubes A, B and C had each reached the normal population for this broth but D had not increased after the first four hours.

This inhibiting effect exerted by a culture on itself and on other cultures is well known. The accelerating factor is apparently specific or at least *Str. lactis* does not accelerate *Es. coli*. This is shown in table 2. In this experiment duplicates of tubes A,

TABLE 2  
*The inhibiting effect of Str. lactis on Es. coli*

AGE hours	STR. LACTIS IN FLASK	TUBE	ES COLI IN FILTERED BROTH				
			4 hours	6 hours	8 hours	10 hours	27 hours
0		A	99,000,000	450,000,000	855,000,000	1,045,000,000	1,250,000,000
4	305,000	B	91,000,000	435,000,000	1,195,000,000	935,000,000	1,085,000,000
8	7,950,000	C	2,045,000	132,500,000	960,000,000	855,000,000	1,325,000,000
24	715,000,000	D	4,940,000	13,550,000	39,500,000	84,250,000	438,000,000

TABLE 3  
*Growth of Es. coli in filtered broth*

AGE OF FLASK CULTURE hours	ES. COLI IN FLASK	TUBE	ES COLI IN FILTERED BROTH			
			2 hours	4 hours	6 hours	24 hours
0		A	4,310,000	55,000,000	730,000,000	1,230,000,000
1	210,000	B	4,860,000	8,750,000	595,000,000	1,250,000,000
2	925,000	C	3,330,000	67,000,000	700,000,000	1,300,000,000
3	4,150,000	D	7,545,000	95,000,000	700,000,000	1,195,000,000
4	17,550,000	E	6,670,000	121,500,000	730,000,000	1,300,000,000
7	660,000,000	F	2,460,000	5,950,000	25,700,000	282,000,000
24	1,260,000,000	G	11,650,000	30,450,000	38,950,000	103,000,000

B, C, and D, as shown in table 1, were inoculated with *Es. coli*. A distinct inhibiting effect is evident from the beginning in tubes C and D. In tubes A, B, and C the inhibiting action was overcome and a normal population finally reached. In D, while an increase continued for twenty-seven hours, the population was still less than half of the normal.

What has been said of the accelerating effect of *Str. lactis* on itself evidently does not apply to *Es. coli*. In table 3 are shown



results obtained by growing *Es. coli* in tubes of broth removed from a culture by filtration at successive stages of growth.

The limiting effect after the growth in the flask had reached some hundreds of millions is evident, but there is no indication of an accelerating effect. The variations in the counts at two and four hours are not consistent and are probably only the differences which could be expected in a set of duplicate flasks. This conclusion was confirmed by results obtained in other experiments of a similar nature.

Table 4 indicates that not only does *Es. coli* have no accelerating effect on *Str. lactis* but that the inhibiting effect is not marked. It is not surprising that such a difference between these cultures should be found. The lactics, while growing rapidly under

TALBE 4  
*The effect of Es. coli on the subsequent growth of Str. lactis*

AGE OF FLASK CULTURE	ES. COLI IN FLASK	TUBE	GROWTH OF STR. LACTIS IN FILTERED BROTH			
			3 hours	7 hours	11 hours	24 hours
<i>hours</i>						
0		A	1,485,000	11,450,000	191,000,000	710,000,000
3	695,000	B	1,010,000	8,350,000	130,500,000	780,000,000
8	143,500,000	C	610,000	10,400,000	309,000,000	740,000,000
27	755,000,000	D	1,225,000	11,300,000	14,700,000	580,000,000

favorable conditions, are sensitive to deficiencies in the nutrient medium. *Es coli*, on the other hand, grows luxuriantly under conditions which would not support the growth of many bacteria. It does well in a very simple medium.

It is possible to explain the accelerating and limiting effects observed in these cultures on nutritional grounds. It is conceivable that through a digesting effect a medium would become more favorable to initial growth, while at the same time some essential food constituent would become partially exhausted so that the normal population could not be attained. It is surprising, however, that through this action cultures of the lactic type should have such a decided effect on a vigorous species like *Es. coli*. while this organism has so little effect of *Str. lactis*.

In the experiments in which a culture in collodion sac was suspended in a flask of broth, the broth in the flask showed the same effect of stimulating the initial growth and restricting the final growth. If this limitation of the final population was due to the partial exhaustion of one or more essential constituents of this broth this could only have taken place through their diffusion into the sac. If this had happened we would expect an increased population in the sac. This did not occur.

The combined stimulating and inhibiting effect observed in the lactic cultures has some resemblance to the phenomena demonstrated by Carrel and Eberling (1923) who showed that the serum of a young animal contains a principle precipitated by CO<sub>2</sub> which increases the proliferative activity of homologous fibroblasts while the serum of old animals has an inhibiting effect only.

The application of the results obtained with the filtered cultures to the growth in the tube is not clear. It is possible that the growth of the lactic culture may have been influenced by the alternating stimulating and inhibiting effect. However, the coli culture showed even more marked variation in rate of growth than the lactic, but if it possessed any self-stimulating action it was so slight that it was not detected.

If we assume that there is an alternation of generations in bacteria a plausible explanation is available. The periods of rapid growth could be looked upon as active vegetative multiplication while the slow periods correspond to a time of sexual regeneration.

It may also be possible to explain these variations by assuming periods of active motility alternating with periods in which there was little or no activity. *Es. coli* which is an active motile organism traveled more rapidly through the tube and showed greater variations in rate of growth. *Str. lactis* is generally considered to be immotile but we have had cultures in the laboratory which were actively motile. Ellis (1920) was able to demonstrate motility with various cocci, including streptococci, and believed that all cocci have cilia. In cultures which were ordinarily immotile, motility could be induced by rapid transfer

on a favorable medium. This condition would be stimulated in the growth through the tube.

#### SUMMARY

Cultures of *Es. coli* and *Str. lactis* inoculated into broth in a tube about 15 meters long grow through the tube in alternating periods of slow and rapid growth.

Filtered cultures of *Str. lactis* stimulate the initial growth of *Str. lactis* but limit the final population.

Filtered cultures of *Str. lactis* do not stimulate the initial growth of *Es. coli* but have a marked limiting effect on the final population.

*Es. coli* does not stimulate itself or *Str. lactis*, and has only a comparatively small effect on the final population of *Str. lactis*.

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# THE ISOLATION OF A BACTERIOLYTIC PRINCIPLE FROM THE ROOT NODULES OF RED CLOVER

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This investigation was suggested by the fact that Gerretsen *et al.* in 1923 reported the isolation of a bacteriophage from the root nodules of clover, lupine, and serradella, and also that the dissolution of the bacteria in the nodules of leguminous plants might be due to the action of the bacteriophage. The bacteriophage was specific in its action for the bacteria of the species of leguminous plants from which the lytic agent was isolated, i.e., the phage isolated from clover nodules produced only a slight action upon *Rhizobia* from common beans and none whatever upon *Rhizobia* from lupine. The phage from serradella failed to produce lysis of the nodule bacteria from lupine, vetch, peas, clover and sanfoin. Gerretsen and his associates were able to isolate the phage from the roots and stems of the leguminous plants as well as from garden and field soil. The phage resisted heating for fifteen minutes at 65°C. and was quite resistant to drying and ultra-violet light. Attempts to produce lysis of the homologous organisms on agar plates were unsuccessful, although an inhibition of growth was noted.

Later Grijns in 1926 and 1927 was able to produce lysis on solid media by employing meat extract-sucrose agar which decreased the production of slime by the nodule organisms. He concluded that leguminous plants do not produce the phage and that the phage does not influence nodule formation or the dissolution of the contents of the nodule.

Israily (1926) in commenting on the work of the above investigators states that it may have significance in explaining the immunity of the leguminous plants to the root nodule organisms.

## EXPERIMENTAL

After several unsuccessful attempts to confirm the results of Gerretsen, Grijns, *et al.* the author has been able to isolate a bacteriolytic principle from red clover nodules which is active against one strain of the bacteria from red clover.

In the isolation of this bacteriolytic principle the following procedure was employed: Six nodules were selected from each plant, washed thoroughly in tap water and treated with  $\text{Hg}(\text{Cl})_2$  solution (1:1000) for fifteen minutes. The nodules were then thoroughly washed with sterile distilled water and collectively crushed in a few drops of yeast water broth. Isolations from these crushed nodules were made on yeast infusion agar. The macerated nodules were then transferred to a tube of yeast infusion free of sugar and incubated at  $28^\circ\text{C}$ .

*Yeast infusion*

$\text{K}_2\text{HPO}_4$ .....	0.5 gram
$\text{MgSO}_4$ .....	0.2 gram
$\text{NaCl}$ .....	0.2 gram
$\text{CaCO}_3$ .....	2.0 grams
Yeast water (10 per cent). ....	200 cc.
Distilled water .....	800 cc.

The medium was autoclaved at 15 pounds for thirty minutes, then filtered through several thicknesses of filter paper until clear; adjusted to pH 7.6, tubed and sterilized. After sterilization the media showed a slight turbidity, but cleared upon standing. After five days this culture was filtered through a Berkefeld filter (porosity *fine*) and the filtrate tested for sterility. Five cubic centimeters of this filtrate were added to 20 cc. of a twenty-four-hour-old liquid culture of the homologous organism. This tube was then incubated for ten days at  $28^\circ\text{C}$ . after which it was filtered, and the filtrate, after testing for sterility, was added to a twenty-four-hour-old culture of the homologous organism. Serial transfers were carried out in this manner decreasing the amount of the filtrate added with each successive transfer.

By employing the above technic in several experiments with nodules from red clover plants, a tube in one experiment showed a decided inhibition of growth in the fourth serial transfer. In the

fifth transfer the turbidity cleared up completely after twenty-four hours incubation. This culture, hereinafter designated the sensitive strain, has been carried through 20 serial transfers, decreasing the amount of filtrate added until in the twentieth transfer, a clearing resulted in twenty-four hours with the addition of one loopful of the nineteenth filtrate to 10 cc. of a twenty-four-hour-old culture of the homologous organism. There was no evidence of lysis of the homologous organism in any of the other experiments.

#### DISCUSSION

In the course of this work, numerous nodules taken at various ages from pea, bean, sweet clover and alfalfa plants as well as those from other red clover plants, have been examined as to the presence of a lytic agent without success. Although Gerretsen suggests that the phage may be active in bringing about the dissolution of the bacteria in the nodule, the author has repeatedly failed to isolate a lytic principle from numerous nodules in which the bacteria were evidently undergoing dissolution as evidenced by the difficulty in isolating the organisms from them. Such nodules were discolored, watery, and ruptured easily. Stained preparations made directly from these nodules have shown very few bacteria.

#### *Certain characteristics of the lytic agent*

Attempts to increase the potency of the lytic principle by further serial transfers have not been successful. With the technic employed the limits of potency lie between  $1 \times 10^{-4}$  and  $1 \times 10^{-5}$ . According to the scheme of d'Herelle (1926) the principle would be classified as weak. Whether this is due to a loss in filtration cannot be stated at this time. Todd (1927) claims that the phage for the Shiga organism has a negative charge and that when the filtration of a lysed culture takes place within the range pH 5.0 to 7.6 there is little or no loss. Rivers (1928) states that filters of kieselguhr, such as the Mandler and Berkefeld types, have negative charges. Since the reaction of these lysed solutions was never below pH 7.0 there should have

been little loss due to absorption by the filter particles in the amount of liquid filtered, usually 10 cc. d'Herelle (1928) suggests that the inability to increase the potency of the filtrate by further serial transfers may be due to the fact that the homologous organism is inherently somewhat resistant to the lytic agent, since he has found that lysable strains vary in their sensitivity to the agent.

The lytic principle is heat labile, a temperature of 70°C. for five minutes serving to inactivate it, as shown by the following experiment. Three 750 cc. flasks containing 200 cc. of yeast infusion broth were inoculated with 1 cc. of a twenty-four-hour-old culture of the sensitive strain of the red clover organism. After twenty-four hours incubation, at which time a definite turbidity had developed, the flasks were treated as follows:

Flask I—2 cc. unheated filtrate added

Flask II—2 cc. heated filtrate added

Flask III—bacteria only

Flask IV—uninoculated control

After twenty-four-hours incubation flask I was completely cleared while flask II showed no evidence of clearing. Upon further incubation secondary growth developed in flask I. Usually within two days, growth again developed in the lysed cultures and they became as turbid as the cultures containing the bacteria but no filtrate.

#### *Strains resistant to lysis*

Cultures isolated from tubes showing secondary growth were inoculated into the yeast infusion media. After twenty-four hours incubation a small portion of an active filtrate was added and the tubes again incubated. These tubes were observed over a period of two weeks without any evidence of lysis.

Serial transfers of the strains resistant to lysis in the presence of the active filtrate have likewise failed to produce any lytic action. When the active filtrate is first added to tubes of yeast infusion broth and the tubes inoculated at once with one drop of a twenty-four-hour-old culture of the sensitive strain, there is a marked inhibition of growth. Visible growth is delayed for two

days when compared with the tubes containing the sensitive strain only, but upon continued incubation the growth is quite rapid. Cultures isolated from these tubes have invariably resisted the lytic action of a filtrate active against the sensitive strain. Experiments designed to determine the effect of the development of resistant strains upon the lytic principle were conducted by adding 0.1 cc. of an active filtrate to several tubes of a twenty-four-hour-old culture of the sensitive strain in yeast infusion broth.

Following the lysis of the culture and the development of secondary growth, the contents of the tubes were filtered at various times and the potency of the filtrate tested against the sensitive strain in serial dilutions. Even after ten days growth in the presence of the secondary cultures there was no apparent loss in the potency of the lytic agent in the filtrate as compared with the filtrate taken at the time of lysis. Rivers (1928) reports the failure of resistant organisms to bind the bacteriophage.

Although the resistance of secondary cultures has been attributed to the production of slime, which offers a specific protection, Grumbach and Dimtza (1927), this could not be true with the resistant strain reported in this paper, since there is no apparent difference in the amount of slime produced by the sensitive and resistant strains.

According to Bronfenbrenner and Korb (1925) secondary cultures ordinarily lose their resistance to phage in a relatively short time, especially if transferred frequently. We have carried our resistant strain over a period of eight months with frequent transfers without any loss in the resistance to lysis.

#### *Progressive rate of lytic action*

In order to determine the rate of lytic action and the effect on diminution of the bacteria in the culture, a series of experiments was conducted in which the following procedure was employed: Tubes containing 15 cc. of yeast infusion broth were inoculated with the sensitive strain and allowed to incubate until slightly turbid. The tube cultures were then treated as follows: 0.1 cc. of an active filtrate was added to tube 1, and to tube 2, 0.1 cc. of



the filtrate inactivated by heat. Numerical counts of the organisms in the tubes were then made by plating out on yeast infusion agar at once and at various intervals during the period of lysis. The results of three typical experiments are given in table 1.

In the three experiments cited it will be noted that the organisms were not completely destroyed, although after twenty-four to thirty hours incubation no turbidity was visible in the tubes containing the active filtrate. Plate counts made at this time showed the minimum number of viable organisms. Upon further incubation the cultures became quite turbid with a corresponding

TABLE 1  
*Rate of destruction of bacteria by the lytic agent*  
(Numbers of bacteria per cubic centimeter in thousands)

TIME	EXPERIMENT I		EXPERIMENT II		EXPERIMENT III	
	Tube 1, filtrate unheated	Tube 2, filtrate heated	Tube 1, filtrate unheated	Tube 2, filtrate heated	Tube 1, filtrate unheated	Tube 2, filtrate heated
Beginning	22,000	30,000	15,000	14,000	18,000	20,000
5 hours	30,000	31,000			21,000	22,000
11 hours	18,000	40,000	4,000	18,000	11,000	24,000
24 hours	110	38,000	30	19,000	180	32,000
30 hours	100	40,000	56	22,000	60	33,000
44 hours	1,200	44,000	5,000	32,000	1,100	42,000
72 hours	10,000	60,000			22,000	58,000
168 hours			180,000	80,000	110,000	72,000

increase in the number of bacteria. After seven days the bacteria per cubic centimeter in the tubes containing the active filtrate greatly exceeded those in the control tubes. Since only cultures resistant to lysis were isolated from such tubes, it may be possible that the rapid secondary growth is due to a stimulation of the resistant strains in the presence of the lytic agent.

Plate counts made during the period of active lysis (twenty-four to thirty hours) contained numerous abnormal colonies. This phenomenon was noted only in those colonies which were growing at the bottom of the plate. Surface colonies developed in a normal manner. The colonies exhibiting this abnormal appearance looked somewhat moth eaten. In many, an entire

sector was dissolved, while in others the clear area was limited to the center of the colony. Quite frequently these areas radiated out from the center, so that the center and periphery appeared normal, while between these two points the clear areas appeared as narrow bands or canals. Israily (1926) describes similar canals in his work with *B. tumefaciens*. Transfers made from these clear zones have failed to promote lysis of the sensitive strain.

### *Specificity of the lytic principle*

In a series of experiments in which an attempt was made to produce lysis of stock cultures of the red clover nodule bacteria by means of the filtrate active against the sensitive strain, only negative results were obtained. Four stock cultures of the red clover organism of known purity from the University of Wisconsin collection, as well as other cultures isolated by the author directly from red clover nodules, have been repeatedly cultured in yeast infusion in the presence of the active filtrate without any evidence of lysis. Attempts to develop sensitive strains by "training" these cultures according to the method of Shwartzman (1927) by repeated cultivation and filtration in the presence of the active principle have likewise met with failure. Gerretsen *et al.* (1923) report that a strain of the serradella organism obtained from the Pribram collection in Vienna was not lysed by their serradella phage. The specificity of the active lytic agent isolated by us was further demonstrated in experiments with the nodule organisms from peas, beans, alfalfa, sweet clover, and vetch where there was no evidence of lysis in any case.

### *Attempts to produce lysis on solid media*

Several experiments were conducted in an attempt to demonstrate lysis of the sensitive strain on solid media. In one series, yeast infusion agar plates provided with porous covers were prepared and on one-half of the plate 0.5 cc. of an active filtrate was evenly smeared. The plates were incubated at 40°C. until the surface moisture had evaporated and inoculated with the sensitive culture by drawing a loopful of the culture across the plate.

After incubation, there was no apparent difference between the growth on the side containing the filtrate and that containing no filtrate. In another series the procedure followed by Koser (1927) for demonstrating lytic action on solid media was followed. Agar slants were inoculated with the sensitive strain and after twenty-four hours incubation one drop of an active filtrate was allowed to flow down the center of the slant and the tubes further incubated. No evidence of plaques or lytic areas was found by this procedure. To plates of yeast infusion agar, smeared evenly with the sensitive strain and incubated twenty hours until visible growth developed, loopfuls of an active filtrate were added at various points on the plates following the procedure of Burnet (1927). Upon further incubation there was a slight inhibition of growth in the presence of the filtrate, but after three days this effect was no longer evident and the growth appeared normal. Attempts to verify the work of Grijns (1927) in producing lysis by cultivation on meat extract-sucrose agar have likewise failed. The sensitive organism grew very slowly and meagrely on this medium.

#### *Plant passage experiments*

Both the sensitive and the resistant strains produce nodules on red clover plants grown in a modified Bryan's-nutrient agar. In order to determine whether or not these strains maintained their characteristics toward the lytic agent, isolations were made from several nodules from the plants inoculated with the sensitive and resistant strains. The cultures were grown in yeast infusion in the presence of the lytic agent. All cultures isolated from nodules produced by the resistant strain were resistant to the agent, whereas each culture isolated from the nodules produced by the sensitive strain was lysed.

In order to determine the effect of further plant passage upon the sensitivity or resistance of the organisms isolated in the first plant passage to the lytic agent, the following jar experiment was conducted. To 10 one-half gallon jars filled with quartz sand and sterilized, a sufficient amount of a modified Bryan's-nutrient solution was added to provide the necessary plant food.

The jars were then planted with sterilized red clover seeds. (a) Three jars were inoculated respectively with three sensitive strains isolated from nodules from the first plant passage. (b) Three jars were inoculated with three resistant strains. (c) Two jars were uninoculated. When the plants were two months old, two plants were removed from each jar of series a and b and ten cultures were isolated from as many nodules. These cultures were then tested for sensitivity or resistance to the lytic agent with the following results: The thirty cultures isolated from the nodules produced by the sensitive strain were lysed by the active filtrate, while the thirty cultures from nodules produced by the resistant strains again resisted lytic action.

Cultures were again isolated from the plants when six months old. The thirty cultures isolated from the nodules produced by the sensitive strain again were lysed by the active filtrate, but of the thirty cultures from nodules produced by the resistant strains only 25 per cent resisted lysis.

A third plant passage experiment in which the seeds were inoculated with resistant and sensitive strains isolated from the second plant passage was conducted in a manner similar to the previous experiment. Cultures isolated from six weeks old plants reacted in the following manner to the lytic agent: 20 cultures isolated from nodules produced by the sensitive strain were lysed by the filtrate while 12 cultures from nodules produced by the resistant strain resisted the lytic action. Due to excessive heat in the greenhouse the plants died so that further isolations were prevented.

These preliminary results suggest that prolonged association with the host plant within the nodule may cause the resistant strains to lose their resistant characteristics and thus become susceptible to lysis by an active filtrate.

#### SUMMARY

1. An agent has been isolated from the root nodules of red clover which produces lysis of a strain of the red clover nodule bacteria isolated from these nodules.

2. The lytic agent has been carried through twenty serial

transfers and filtrations in the presence of its homologous organism without any loss in activity. However, the potency of the filtrate has failed to increase markedly during these serial transfers.

3. The agent is specific for its own homologous strain and fails to produce lysis of stock strains of the red clover nodule bacteria as well as of strains isolated from peas, beans, alfalfa and vetch.

4. Upon continued incubation of the lysed culture, secondary growth develops. Bacteria, isolated from such secondary growth, resist the lytic action of the agent.

5. Attempts to produce lysis on solid media by streaking the sensitive organisms and agent on the surface of agar plates have failed.

6. The sensitive and resistant strains both exhibit the characteristics of the red clover nodule bacteria and produce nodules on red clover.

7. Isolations made from nodules from young plants produced by these respective strains reveal only the homologous strain. Results are submitted which may indicate that the resistant characteristics are lost upon prolonged growth within the nodule.

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# THE INFLUENCE OF CERTAIN INORGANIC SALTS ON THE GERMICIDAL ACTIVITY OF HYDROGEN PEROXIDE

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Several investigators have noted during recent years that the decomposition of hydrogen peroxide is accelerated by the presence of certain inorganic salts. The results of earlier investigations have been summarized by Bohnson (1921) who studied the mechanism of the decomposition of hydrogen peroxide by ferric salts, and noted that ethyl alcohol was oxidized to acetic acid by hydrogen peroxide in the presence of a ferric salt, a reaction which was quantitatively studied by Walton and Christensen (1926). Walton and Graham (1928) in a study of the oxidation of certain dicarboxylic acids showed that ferric or cupric salts increased the oxidizing power of hydrogen peroxide. Promoter action<sup>1</sup> in the catalytic decomposition of hydrogen peroxide has been extensively studied by Professor J. H. Walton and his students. Bohnson and Robertson (1923) and Robertson (1925) studied the promotor effect of cupric salts or the decomposition of hydrogen peroxide catalyzed by ferric salts. Robertson (1926, 1927) showed that the decomposition of hydrogen peroxide catalyzed by potassium dichromate was promoted by cobaltous or manganous salts. Walton and Graham (unpublished data) found that the oxidation of hydrazine by hydrogen peroxide was accelerated by both cupric and ferric ions, and that the cupric ions promoted the oxidation catalyzed by the ferric ions.

The germicidal activity of hydrogen peroxide is apparently

<sup>1</sup> A promoter in the broadest sense is a substance whose presence in relatively small amount increases the activity of a catalyst.



due to its oxidizing action, and, if this is true, its effectiveness should be materially increased by any substance which increases its oxidizing power. The present investigation was undertaken to test the accuracy of this hypothesis.

#### TECHNIQUE

A modification of the Hygienic Laboratory method (1912) of determining the phenol coefficient was used in measuring the germicidal effect of the hydrogen peroxide with different concentrations of the inorganic salts. Twenty-four-hour cultures of *Es. coli* or *Staph. aureus* were used as test organisms. One-half cubic centimeter of the twenty-four-hour culture was introduced into 9.5 cc. of the disinfectant which was adjusted to a pH of 6.4. Transfers were made with a 4 mm. loop from the disinfectant to the liquid media at intervals of two and one-half minutes during the period of the tests, which were conducted at room temperature.

The medium used for this study had the following composition:

Liebig meat extract.....	5 grams	} + 1.5 per cent agar for slants
Pepton (Armour) .....	10 grams	
NaCl.....	5 grams	
H <sub>2</sub> O.....	1000 grams	

The pH of the medium was adjusted to 6.8.

In order to observe the effect of organic materials, such as carbohydrates and proteins, upon the disinfectant, the following additional experiments were made.

Sterile pieces of number two Whatman's filter paper were immersed in a twenty-four-hour broth culture of the organism and then transferred into tubes of the disinfectant. From this tube they were transferred at intervals of five minutes to a wash tube of sterile broth, and from this to another tube of sterile broth in which they were incubated for forty-eight hours.

A two per cent solution of gelatin was prepared with which the hydrogen peroxide was diluted for the experiments. The organisms were tested in the gelatin dilutions in the same manner as outlined before.

The inorganic salts used were all of C.P. quality which were

recrystallized at least once, while the hydrogen peroxide was of commercial quality. Its concentration was checked by titration with a standard solution of potassium permanganate and it was found to contain 2.5 per cent by weight of  $\text{H}_2\text{O}_2$ . All dilutions were made with distilled water.

## EXPERIMENTAL

*Hydrogen peroxide alone*

The germicidal activity of hydrogen peroxide on *Es. coli* and *Staph. aureus* was determined in accordance with the method described. The results are summarized in table 1. The data

TABLE 1  
Germicidal activity of hydrogen peroxide on *Es. coli* or *Staph. aureus*

DILUTION OF 2.5 PER CENT $\text{H}_2\text{O}_2$ WITH WATER	TIME IN MINUTES									
	2.5	5	7.5	10	12.5	15	20	25	30	45
1/1	+	—	—	—	—	—	—	—	—	—
1/2.5	+	+	+	+	—	—	—	—	—	—
1/5	+	+	+	+	+	—	—	—	—	—
1/7.5	+	+	+	+	+	±	—	—	—	—
1/10	+	+	+	+	+	+	—	—	—	—
1/20	+	+	+	+	+	+	+	—	—	—
1/30	+	+	+	+	+	+	+	+	+	+

+ indicates growth, — indicates absence of growth.

given in this and all subsequent tables represent the average of several checks.

When either *Es. coli* or *Staph. aureus* was treated with a one to one dilution of the stock solution of hydrogen peroxide, the organisms were killed within a period of five minutes. Higher dilutions of the hydrogen peroxide decreased the toxicity very markedly until one part of hydrogen peroxide to thirty parts of water exhibited no toxicity during a period of forty-five minutes.

*Hydrogen peroxide with cupric and ferric sulphates*

The combined effect of ferric and cupric sulphates on the germicidal activity of hydrogen peroxide was determined when the

concentration of each salt was equivalent to 0.1 of a millimol of the metallic ion per 120 cc. of solution. The results are given in table 2 and figure 1. The effect of the ferric and cupric ions is pronounced. In their presence, a dilution of the hydrogen peroxide of 1-50 is equivalent in toxicity to a dilution of 1-1 of hydrogen peroxide alone.

The concentrations of both the ferric and cupric ions were varied and their effect determined. The optimum concentrations of ferric and cupric ions were found to be 0.1 of a millimol per

TABLE 2

*The effect of cupric and ferric sulphates on the germicidal activity of hydrogen peroxide on Es. coli or Staph. aureus*

Concentration of salts— $\text{Fe}^{+++}$  0.1 millimol per 120 cc.;  $\text{Cu}^{++}$  0.1 millimol per 120 cc.

DILUTION OF 2.5 PER CENT $\text{H}_2\text{O}_2$ WITH WATER	TIME IN MINUTES									
	2.5	5	7.5	10	12.5	15	20	25	30	45
1/50	±	—	—	—	—	—	—	—	—	—
1/100	+	+	±	—	—	—	—	—	—	—
1/200	+	+	+	+	—	—	—	—	—	—
1/300	+	+	+	+	+	—	—	—	—	—
1/325	+	+	+	+	+	+	—	—	—	—
1/350	+	+	+	+	+	+	+	+	—	—
1/375	+	+	+	+	+	+	+	+	+	+
$\text{Fe}_2(\text{SO}_4)_3$ alone	+	+	+	+	+	+	+	+	+	+
$\text{CuSO}_4$ alone	+	+	+	+	+	+	+	+	+	+
$\text{Fe}_2(\text{SO}_4)_3 + \text{CuSO}_4$	+	+	+	+	+	+	+	+	+	+

120 cc. of solution. When present in concentrations of 0.01 of a millimol per 120 cc. of solution, their influence had entirely disappeared. Concentrations of 0.25 of a millimol per 120 cc. of solution gave a slight increase in toxicity over hydrogen peroxide alone. When the concentrations of both ions were increased, the toxicity of the peroxide gradually diminished. At concentrations of 0.1 molar (100 millimolar), both the ferric sulphate and cupric sulfate alone were toxic to *Es. coli* and *Staph. aureus*.

In a concentration of 0.1 millimol per 120 cc. of solution, neither ferric sulfate nor copper sulfate, used singly with hydrogen peroxide, was effective in increasing its toxicity.

In order to test the hydrogen peroxide under conditions which would more nearly approximate those which it would meet in actual use, the filter paper test and the gelatin dilution tests

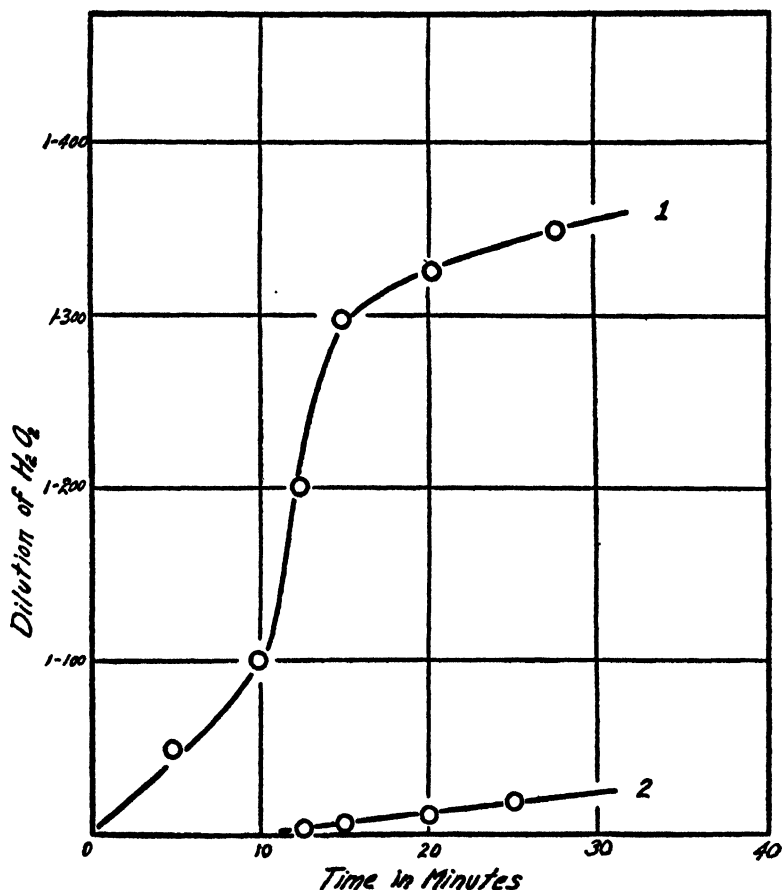


FIG. 1. GERMICIDAL ACTIVITY ON *ESCHERICHIA COLI*  
1,  $H_2O_2$  plus  $Fe^{+++}$  and  $Cu^{++}$ ; 2,  $H_2O_2$  alone

were made. The results are summarized in tables 3 and 4. In the filter paper tests it was observed that the carbohydrate material had little effect upon the toxicity of the hydrogen peroxide for either organisms. The gelatin dilution tests showed

that the germicidal activity of the hydrogen peroxide was destroyed in part by the protein material.

*Hydrogen peroxide with potassium dichromate and manganous or cobaltous sulphates*

The influence of potassium dichromate promoted by manganous sulphate or cobaltous sulphate on the germicidal activity

TABLE 3

*Filter paper tests on Escherichia coli and Staphylococcus aureus*

Concentration of salts— $\text{Fe}^{+++}$  0.1 millimol per 120 cc.;  $\text{Cu}^{++}$  0.1 millimol per 120 cc.

DILUTION OF 2.5 PER CENT $\text{H}_2\text{O}_2$ WITH WATER	ESCHERICHIA COLI			STAPHYLOCOCCUS AUREUS		
	5 minutes	10 minutes	15 minutes	5 minutes	10 minutes	15 minutes
1/50	+	—	—	+	—	—
1/100	+	—	—	+	±	—
1/200	+	+	+	+	+	—
1/300	+	+	+	+	+	+

TABLE 4

*Gelatin dilution tests on Es. coli or Staph. aureus*

Concentration of salts— $\text{Fe}^{+++}$  0.1 millimol per 120 cc.;  $\text{Cu}^{++}$  0.1 millimol per 120 cc.

DILUTION WITH GELATIN SOLUTION	TIME IN MINUTES									
	2.5	5	7.5	10	12.5	15	20	25	30	40
1/50	+	+	+	+	—	—	—	—	—	—
1/100	+	+	+	+	+	+	—	—	—	—
1/200	+	+	+	+	+	+	+	—	—	—
1/300	+	+	+	+	+	+	+	+	+	+

of hydrogen peroxide was also studied and found to be comparable to the combined effect of cupric and ferric sulphates. The increase of toxicity which resulted was practically the same in the higher concentrations of the hydrogen peroxide as in the case of the iron and copper catalysis, but was considerably lower as the concentration of the hydrogen peroxide was decreased. The results are tabulated in tables 5 and 6.

[illegible]

*Phenol Coefficients*

After the germicidal activity of phenol was determined (table 7) the phenol coefficients were calculated in accordance with the method formulated by Reddish (1927)

$$\text{Phenol Coefficient} = \frac{\text{Dilution of X which will kill in 10 minutes but not in 5 minutes}}{\text{Dilution of phenol which will kill in 10 minutes but not in 5 minutes}}$$

Using this formula the following phenol coefficients were secured with *Es. coli*:

Hydrogen peroxide alone .....	0.014
Hydrogen peroxide plus ferric and cupric sulphates.....	1.4
Hydrogen peroxide plus potassium dichromate and cobaltous sulphate or manganous sulphate.....	1.4

With *Staph. aureus*:

Hydrogen peroxide alone.....	0.012
Hydrogen peroxide plus ferric and cupric sulphate .....	1.2

## SUMMARY

1. The germicidal activity of hydrogen peroxide on *Es. coli* and *Staph. aureus* is greatly increased by the combined presence of ferric and cupric sulfates.

2. The optimum concentrations of ferric and cupric sulfates were found to be approximately one tenth of a millimol of each of the metallic ions per one hundred and twenty cubic centimeters of solution.

3. The influence of these salts on the toxicity of hydrogen peroxide is not altered by the presence of cellulose, but is slightly decreased in the presence of protein matter.

4. Potassium dichromate when promoted with manganous sulfate or cobaltous sulfate has been found to increase the toxicity of hydrogen peroxide for *Es. coli* approximately as much as the ferric and cupric sulfates. The effects of potassium dichromate with manganous sulfate and potassium dichromate with cobaltous sulfate have been measured at only one concentration, namely one-tenth of a millimol of the dichromate and metallic ion per one hundred and twenty cubic centimeters of solution.

5. The phenol coefficients of hydrogen peroxide when measured in relation to *Es. coli* and *Staph. aureus* were 0.014 and 0.012 respectively, but in the presence of the optimum concentrations of cupric and ferric sulphates they were increased to 1.4 and 1.2; while 0.1 of a millimol of dichromate ions in 120 cc. of solution in the presence of the same concentration of cobaltous or manganous ions increased the phenol coefficient of hydrogen peroxide measured on *Es. coli* to 1.4. .

6. Since the salts which catalyze and promote the decomposition and increase the oxidizing property of hydrogen peroxide all increase the germicidal activity of hydrogen peroxide, it seems logical to conclude that the toxicity of hydrogen peroxide is dependent upon its ability as an oxidizing agent.

The writers desire to express their appreciation to Professor J. H. Walton for suggesting the problem involved in this investigation.

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## FILTRATION OF BACTERIOPHAGE

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Problems concerning filtration are constantly assuming a greater significance in bacteriology and in immunology. A most encouraging aspect of this situation is that the idea of adsorption is now generally accepted whereas, until quite recently, the mechanical rôle of filters in removing bacteria was the only factor considered. This has led to a considerable advance in our knowledge of the nature of the materials filtered. In the study of so-called filterable viruses and of bacteriophage in particular the rapid advance in understanding of filters has been productive of some outstanding achievements. Among these has been the demonstration by Kramer (1927) that by use of materials of positive charge, such as plaster of Paris, substances which passed the usual negatively charged filters could be removed from suspension. In the study of bacteriophage it may readily be seen that much use might be made of such findings. Problems relating to the nature of the bacteriophage as well as to its therapeutic application are dependent to a considerable extent upon filtration. Such questions as the relationship between the bacteriophage and the "dissolved" bacterial proteins, the size of the bacteriophage particle, and the charge which this particle carries, may well utilize filtration as a possible means of solution. Qualitative and quantitative changes in bacteriophage as a result of filtration have engaged the attention of many investigators, and Todd (1927), who demonstrated that in acid reaction bacteriophage is inhibited in its passage through a Berkefeld filter, has suggested a type of research which should yield much information. We have attempted to verify Todd's work and to extend it.

EFFECT OF H-ION CONCENTRATION ON FILTRATION OF  
BACTERIOPHAGE*Mandler Filters*

The usual reaction for the filtration of bacteriophage is on the alkaline side of neutrality since the material is prepared in broth at a hydrogen-ion concentration of pH 7.6 to 7.8. The question naturally arises then, as to the possibility of changing the amount of bacteriophage in filtrates by changing the hydrogen-ion concentration. There are known limits to such a procedure for the principle is inactive at pH 4.0 and 11.0. It is the generally accepted opinion that the principle has a negative charge. Hence, as the reaction of the medium becomes acid, it might be expected that the bacteriophage would assume a positive charge and be retained by the filter which formerly allowed it to pass. Conversely, increase in alkalinity of the medium might result in an increase in the amount of bacteriophage passing through the filter. In brief, the problem concerns the optimum hydrogen-ion concentration for maximum yield of bacteriophage. Todd (1927) has shown that an acid reaction reduces the bacteriophage yield. At a hydrogen-ion concentration of pH 5.0, filtration of a particular bacteriophage race was inhibited. Whether this result holds for all bacteriophage races remains to be shown. For this reason we utilized two races, certainly different from Todd's and quite different from each other. Both were polyvirulent races, one active against gram-negative bacilli of the enteric group, the other active against staphylococci. Passage of these races through six Mandler filters of nearly identical pressure tests showed that no bacteriophage was adsorbed by any of the filters when the reaction of the liquid phase was about neutral.

Determination of the amount of protein nitrogen present in the filtrates showed a maximum variation of  $\pm 37$  mgm. In succeeding experiments, differences in excess of this figure are considered significant. The reason for these determinations will appear later.

Bacteriophage active against enteric bacilli was distributed in flasks, to each of which a quantity of hydrochloric acid or sodium

hydroxide was added sufficient to bring the reactions to pH 4.0, 5.0, 6.0, 8.0, 9.0, and 10.0 respectively. The reactions were checked by electrometric methods. About 50 cc. of the contents of each flask was filtered through one of the six Mandler filters mentioned above, and the filtrates were titrated for bacteriophage content according to the method of serial dilution. Plates were made and plaque counts were used to check the readings in the broth. The results are given in table 1.

TABLE 1  
*Typhoid bacteriophage—Mandler filters*

FILTER	pH	LYSIS IN BROTH AT DILUTION						MILLION PLAQUES PER CUBIC CENTI- METER	NITRO- GEN IN GRAMS PER 100 CC.
		10 <sup>-2</sup>	10 <sup>-4</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>	10 <sup>-8</sup>	10 <sup>-9</sup>		
None	4.0	++++	—	—	—	—	—	10	.
A	4.0	—	—	—	—	—	—	10	0.1114
None	5.0	++++	++	—	—	—	—	600	
B	5.0	+	—	—	—	—	—	10	0.1165
None	6.0	++++	++++	++++	++	—	—	1,500	
C	6.0	+++	++++	++	+	—	—	1,100	0.1183
(None)	7.0	++++	++++	++++	++	+	—	1,500	
None	8.0	++++	+++	++	+	—	—	4,500	
D	8.0	++++	++++	++++	++++	+	—	1,600	0.1207
None	9.0	++++	+++	++	+	—	—	3,500	
E	9.0	++++	++++	++++	++++	—	—	1,600	0.1123
None	10.0	++++	++++	++++	++	+	—	8,000	
F	10.0	++++	++++	++++	++	—	—	600	0.1032

In this experiment, the phage used had already been filtered once as a stock preparation. As a check upon these results and in order to determine whether *development* of phage in different reactions would alter the findings, flasks of broth were adjusted to the reactions indicated, inoculated with identical quantities of culture and of bacteriophage, and incubated. Control flasks were inoculated with culture but not with bacteriophage. Lysis occurred to some extent in all flasks. The contents of these flasks were then filtered through the same Mandler filters used in the previous experiment and both filtered and unfiltered lysates were titrated. The results are given in table 2.

Our stock staphylococcus bacteriophage is in many respects strikingly different from the principle active against Gram-negative organisms. Its action is less rapid; it does not permit the development of secondary cultures; and it is in many other particulars a very peculiar and well defined race. Nevertheless, when studied in the same manner as the bacteriophage used in

TABLE 2  
*Typhoid bacteriophage—Mandler filters*

FILTER	pH	LYSIS IN BROTH AT DILUTION						MILLION PLAQUES PER CUBIC CENTI- METER	NITROGEN IN GRAMS PER 100 cc.
		10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>		
None	4.3	++++	++	—	—	—	—	10	0.1405
A	4.3	++++	++	—	—	—	—	10	
None	5.0	++++	++++	+	—	—	—	1,000	0.1426
B	5.0	++++	++	—	—	—	—	100	
None	5.6	++++	++++	++	—	—	—	2,500	0.1442
C	5.6	++++	++++	++	—	—	—	1,200	
None	6.0	++++	++++	+++	+	—	—	2,500	0.1470
D	6.0	++++	++++	++	—	—	—	1,500	
None	6.8	++++	++++	++	—	—	—	3,000	0.1502
E	6.8	++++	++++	+++	—	—	—	2,000	
None	7.5	++++	++++	+++	—	—	—	2,000	0.1568
F	7.5	++++	++++	++	—	—	—	1,000	
None	8.0	++++	++++	++++	+++	—	—	2,500	0.1588
A	8.0	++++	++++	+++	++	—	—	1,200	
None	9.3	++++	++++	++++	+++	—	—	2,000	0.1588
B	9.3	++++	++++	++	+	—	—	750	
None	9.7	++++	++++	+++	+	—	—	300	0.1588
C	9.7	++++	++++	++	—	—	—	150	
None	10.0	++++	+++	++	+	—	—	100	0.1588
D	10.0	++++	+++	+	—	—	—	20	

the preceding experiment, it yields identical results as is shown in the protocol in table 3.

### *Seitz filters*

The above experiments were repeated many times with Mandler and with Seitz filters. The latter were used not only to confirm the results obtained with Mandler filters, but to investigate the alleged inferiority of this type of filter for bacteriophage work. Some statements have appeared in the literature relative to the

**TABLE 3**  
*Staphylococcus bacteriophage—Mandler filters*

FILTER	pH	LYSIS IN BROTH AT DILUTION										PLAQUES MILLION PER CUBIC CENTI- METER	NITROGEN IN GRAMS PER 100 CC.
		10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>	10 <sup>-8</sup>	10 <sup>-9</sup>	10 <sup>-10</sup>	10 <sup>-11</sup>		
None	4.5	+++	+++	+++	+++	—	—	—	—	—	—	1.5	0.1750
A	4.5	+++	+++	—	—	—	—	—	—	—	—	0	0.1750
None	5.0	+++	+++	+++	+++	—	—	—	—	—	—	400	0.1768
B	5.0	+++	+++	+++	—	—	—	—	—	—	—	30	0.1768
None	6.0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	600	0.1886
C	6.0	+++	+++	+++	+++	+++	+++	+++	+++	+++	—	600	0.1886
None	7.0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	600	0.1765
D	7.0	+++	+++	+++	+++	+++	+++	+++	+++	+++	—	600	0.1765
None	8.0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	600	0.1723
E	8.0	+++	+++	+++	—	—	—	—	—	—	—	600	0.1723
None	9.0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	600	0.1723
F	9.0	+++	+++	+++	+++	+++	+++	+++	+++	+++	—	600	0.1723
None	10.0	+++	+++	+++	+++	+++	+++	+++	—	—	—	25	0.1648
13	10.0	+++	+++	+++	+++	—	—	—	—	—	—	1	0.1648

TABLE 4  
*Staphylococcus bacteriophage—Seitz filters*

FILTER	pH	LYSIS IN BROTH AT DILUTION						MILLION PLAQUES PER CUBIC CENTI- METER	NITRO- GEN IN GRAMS PER 100 CC.
		10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>		
None	4.5	++++	++++	—	—	—	—	100	0.1730
Seitz	4.5	—	—	—	—	—	—	0	
None	5.0	++++	++++	—	—	—	—	?	0.1740
Seitz	5.0	—	—	—	—	—	—	0	
None	6.0	++++	++++	++++	++++	++++	++++	200	0.1750
Seitz	6.0	++++	++++	++++	++++	++++	—	100	
None	7.0	++++	++++	++++	++++	++++	++++	50	0.1750
Seitz	7.0	++++	++++	++++	++++	++++	—	50	
None	8.0	++++	++++	++++	++++	++++	++++	200	0.1730
Seitz	8.0	++++	++++	++++	++++	—	—	200	
None	9.0	++++	++++	++++	++++	++++	++++	100	0.1700
Seitz	9.0	++++	++++	++++	++++	—	—	6	
None	10.0	++++	++++	—	—	—	—	20	0.1680
Seitz	10.0	—	—	—	—	—	—	0	

TABLE 5  
*Typhoid bacteriophage—Seitz filters*

FILTER	pH	LYSIS AT DILUTION						PLAQUES IN MILLIONS PER CUBIC CENTI- METERS	NITRO- GEN IN GRAMS PER 100 CC.
		10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>		
None	4.5	++++	++++	+++	++	—	—	200	0.6972
Seitz	4.5	++++	—	—	—	—	—	2	
None	7.0	++++	++++	+++	+++	+++	—	3,000	0.7112
Seitz	7.0	++++	++++	++	+	+	—	2,000	
None	10.0	++++	++	+	—	—	—	300	0.6888
Seitz	10.0	+++	+	—	—	—	—	10	

adsorption of bacteriophage by Seitz filters which did not check with daily experience in our laboratory. Gildmeister and Herzberger (1924) specifically claim that Seitz filters allow less lysin to pass than do Berkefeld filters; that they change bacteriophage quantitatively; and that with this type of filter adsorption plays no rôle. The latter statement can definitely be refuted by a simple experiment: Kramer (1927) has shown that negatively charged filters permit the passage of negatively charged dyes and retain those that are positively charged. Thus, a Mandler filter will decolorize Victoria blue and permit the passage of Congo red. Seitz filters behave in exactly the same manner, except that more dye is required to saturate the filter than is required with Mandler filters of equivalent size. Obviously then, specific adsorption is as much a property of Seitz as of Mandler or of Berkefeld filters. The effect of Seitz filters on bacteriophage is shown in the protocols in tables 4 and 5.

These protocols are but two of many similar experiments, all giving equivalent results. The differences between filtered and unfiltered material within the range pH 6.0 to 8.0 are not sufficient to indicate that there is an appreciable loss of lytic principle on filtration. There is much to indicate a qualitative change. Thus if two samples give lysis at the same dilution but the degree of lysis varies as is shown below there is no doubt but that a qualitative and not a quantitative difference exists:

	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-7</sup>	10 <sup>-8</sup>
Unfiltered.....	++++	++++	++++	+++	-
Filtered.....	++++	++++	++	+	-

Such a comparison between filtered and unfiltered phage is practically an invariable result *regardless of the type of filter employed*. Hence, although Gildmeister and Herzberger (1924) are correct in their statement that Seitz filters change phage qualitatively, they cannot be substantiated in their inference that Berkefeld filters do not cause such a change. To be sure we are assuming that in this respect Berkefeld and Mandler filters are alike. If they are not, the criticisms which are directed against Seitz filters must likewise be applied to the Mandler type.



*Plaster of Paris filters*

A continuation of a study of this type would naturally involve an attempt to alter the charge of the filter concerned. As a matter of fact it is quite necessary to determine the extent to which the hydrogen-ion concentrations involved could change the

TABLE 6  
*Typhoid bacteriophage—plaster of Paris filter*

FILTER	pH	LYSIS IN BROTH AT DILUTION						PLAQUES IN MILLIONS PER CUBIC CENTI- METER	NITRO- GEN IN GRAMS PER 100 CC.
		10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>		
None	5.0	++++	++++	++++	++++	++	—	500	0.0756
Plaster	5.0	++++	++++	++++	++	—	—	100	
None	7.0	++++	++++	++++	++++	++	—	700	0.0543
Plaster	7.0	++++	++	—	—	—	—	0	
None	9.0	++++	++++	++++	++++	+	—	100	0.0699
Plaster	9.0	++++	++++	++++	+++	—	—	500	

*Staphylococcus bacteriophage—plaster of Paris filter*

FILTER	pH	LYSIS IN BROTH AT DILUTION								PLAQUES IN MILLIONS PER CUBIC CENTI- METER	NITRO- GEN IN GRAMS PER 100 CC.
		10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>	10 <sup>-8</sup>	10 <sup>-9</sup>		
None	4.5	++++	+++	+++	++	+	+	—	—	600	0.0688
Plaster	4.5	++++	+++	++	+	+	+	—	—	500	
None	7.6	++++	++++	+++	+++	++	+	+	—	2,000	0.0672
Plaster	7.6	+	—	—	—	—	—	—	—	0.8	
None	10.0	+++	+++	++	+	+	+	—	—	400	0.0682
Plaster	10.0	+++	++	+	+	+	—	—	—	180	

charge of the filter. In order to study this, use was made of the phenomenon of electroendosmosis. By the addition of hydrochloric acid to the fluid passing through the filter it was impossible to reverse the charge as shown by a reversal in the direction of the flow of the water, at least within the range of hydrogen-ion con-

centration not destructive of bacteriophage. In the case of the Mandler filters this result was checked by cataphoresis. Finely ground filter particles were suspended in water and on passage of a current of electricity they migrated towards the anode. The addition of acid failed to change the direction of this migration. Thanks to the researches of Mudd (1922) and of Kramer (1927) however it was possible to study the effect of reversal of charge on the passage of bacteriophage by substituting a positively charged filter for the negatively charged Seitz and Mandler. The results are shown in the protocols in table 6.

There are many practical difficulties concerned with the use of plaster of Paris filters. They are relatively less porous than Mandler or Seitz filters; they have smaller pores and consequently filter more slowly. Their action may be hastened by the use of more pressure, but their strength is not sufficient to stand more than a few pounds. They are extremely liable to contain flaws, some very minute, which permit the passage of particles which are otherwise adsorbed by the filter. The use of Congo red or some other negatively charged dye in the material to be filtered serves to indicate the presence of gross leaks. Under most favorable circumstances these filters become quickly saturated and fail to retain positively charged particles. Thus, at pH 7.0, bacteriophage may be absent from the first cubic centimeter of filtrate or even from the first several cubic centimeters depending upon the size of the filter. Sooner or later, however, it appears. Because of these difficulties we feel that our results with these filters are somewhat less reliable than those previously reported. Repeated experiments, however, have sufficiently confirmed the results shown in the two protocols to justify general conclusions.

#### DISCUSSION

A consideration of the charge of the bacteriophage particle, as well as of many problems concerning the nature of the principle, is dependent upon an understanding of the relationship between the bacteriophage and the proteins with which it is associated. The data that we have presented show that the curves for the yield of protein and of bacteriophage are parallel, regardless of the

charge of the filter used. Thus the possibility of mechanical retention of coagulated protein and such bacteriophage as it might hold may be excluded. Whether the bacteriophage is adsorbed to the protein or whether it is affected in an identical manner by change in hydrogen-ion concentration it is impossible to state. No doubt, the use of purified phage such as is described by Weiss (1927) and by Asheshov (1928) would throw considerable light on this question. Our attempts to produce purified phage have not resulted in a product of sufficient concentration to be used in such experiments. We feel however that the results of our investigations are of sufficient interest in themselves to warrant their publication without awaiting the data that might result from the use of such material.

#### CONCLUSIONS

Bacteriophage in passing Mandler and Seitz filters is removed from suspension at hydrogen-ion concentrations of pH 4.5 to 5.0 and pH 9.0 to 10.0.

The behavior of these two filters in this respect is identical.

Both Seitz and Mandler filters have a slight qualitative effect upon bacteriophage although neither affects quantity of phage at a neutral reaction.

Plaster of Paris filters remove bacteriophage from suspensions at a hydrogen-ion concentration of pH 7.0 but permit its passage at pH 4.5 to 5.0 and pH 9.0 to 10.0. They reduce the amount of protein in a similar manner.

Bacteriophage, as well as negatively charged dyes, saturate plaster of Paris filters rapidly.

It appears from these observations that bacteriophage is adsorbed to the protein in the suspensions.

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# THE DETECTION OF AMMONIA PRODUCTION BY BACTERIA IN AGAR SLANTS

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The production of ammonia from various substrata serves, in many cases, as an important character in the identification of species of bacteria. The detection of ammonia as used by Thomas (1912), was first recommended by Ayers, Rupp, and Mudge (1921) and later by Hucker and Wall (1922) as having certain advantages over Nessler's reagent. The reagents necessary in the Thomas test may be used in the presence of glucose, protein, and other organic compounds which generally make up the nutrient media in common use in the laboratory. In the Manual of Methods for Pure Culture Study of Bacteria by the Society of American Bacteriologists the reagents for the Thomas test are listed as follows: I. A 5 per cent solution of phenol. II. A solution of sodium hypochlorite containing 1 per cent available chlorine. To obtain this amount of available chlorine the solution should be so adjusted that 1 cc. of it should neutralize 2.82 cc. of a N/10 solution of sodium thiosulphate (i.e., 24.8 grams to the liter), titrating with starch as an indicator in the presence of acetic acid and potassium iodide.

In carrying out this technic in the laboratory, difficulty is often encountered in maintaining a sodium hypochlorite of the proper concentration of available chlorine. Hypochlorite preparations are rarely stable and gradually undergo changes with the loss of available chlorine. For the Thomas test, stock solutions of chlorinated lime are sometimes used similar to the solutions available as disinfectants. It is necessary to standardize these hypochlorite solutions before use in order to adjust the final solution to the proper concentration. Frequently it is found that

the available chlorine in these solutions has been reduced so much that the solution is useless, as an insufficient amount not only causes inconvenience but has a marked effect upon the results. Thomas has suggested that other oxidizing agents may be of use in place of the hypochlorite. This encouraged the author to try the use of a substitute for the hypochlorite which would be easy to prepare and would not require standardization. After repeated trials such a solution was found to be one containing hypobromite. This reagent is prepared by adding 35 cc. of 2N sodium hydroxide to 100 cc. of saturated bromine water. At first it may seem of no advantage to use such an unstable compound as hypobromite. However, the hypobromite solution is easily made up, and a saturated solution of bromine is sufficiently constant to warrant its use as a routine reagent. The solubility of bromine in grams per 100 cc. water is:

	<i>grams per 100 cc.</i>
At 15°C.....	3.65
At 20°C.....	3.58
At 25°C.....	3.48

As the solubility does not vary greatly at these temperatures a solution of bromine in water at "room temperature" does not need standardization for the purpose under discussion. A solution containing 3.5 grams of bromine in a volume of 135 cc. will titrate per 1 cc. 3.24 cc. N/10 thiosulphate. Acceptable bromine water for the purpose of this test may be made as follows: In a glass stoppered bottle, distilled water is shaken with sufficient bromine to leave an undissolved excess. The bottle is cooled in running tap water. If cold water is used (see table above) no difficulties will arise. Without any special precautions these hypobromite solutions were found to titrate approximately 3.2 cc. Small variations do not affect the results. The sodium hydroxide must of course be added to the bromine water immediately, as otherwise some bromine will escape. The hypobromite reagent is a yellow solution, does not have a strong odor, and keeps for some time. A solution which at first titrated 3.15 N/10 thiosulphate per cubic centimeter showed a titration of 3.05 after three weeks.

In the Thomas test, other phenols may be substituted for the recommended phenol solution. Cresol gives a similar reaction but there appears to be no advantage in using it in place of phenol. However, thymol seems to be of great interest. When a solution of thymol in sodium hydroxide is added to a weak<sup>1</sup> solution of an ammonium salt, mixed well, a hypobromite solution added and the whole left for 20 minutes, a blue color appears which is lighter or darker according to the amount of ammonia present. When a few cubic centimeters of ether are added and shaken with the mixture, the ether absorbs the color with a resultant beautiful red-violet shade. When allowed to stand for a few seconds the ether collects upon the surface of the mixture and makes the reaction very striking. The bromine compounds which are simultaneously formed do not render the reaction indistinct, especially when ether is used.

The mixture may have a green color caused by a mixture of the blue dye from the reaction and the yellow color of the bromine compounds. This green color may indicate a positive reaction, but the reaction appears more definite when the blue color is extracted as mentioned above.

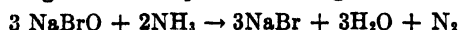
The reagents for this test are:

I. Thymol .....	2 grams
Sodium hydroxide 2 N.....	10 cc.
Water .....	90 cc.
II. Bromine water . .....	100 cc.
Sodium hydroxide 2 N.....	35 cc.

The test may be carried out as follows:

One cubic centimeter of reagent I is added to 5 cc. of the liquid (which must not be acid) to be tested for ammonia and thoroughly mixed. One cubic centimeter of reagent II is then added and shaken and allowed to stand for 15 to 20 minutes, a small amount of ether added, shaken well and allowed to stand until the ether rises to the top. It is important to add the two solutions sepa-

<sup>1</sup> A strong solution gives a development of nitrogen.



rately and not to add the ether before the color has had time enough to develop.

A comparison was made between this technic (D), the Thomas test (A) as described in the Manual of Methods for Pure Culture Study, procedure (B) in which the hypobromite reagent was substituted for the hypochlorite solution, and a procedure (C) in which the thymol solution was used as a first reagent and the original hypochlorite solution as the second solution.

The methods were tried upon 5 cc. of solutions of  $\text{NH}_4\text{Cl}$ ,  $\text{CH}_3\text{NH}_2\text{Cl}$ , and  $\text{NH}_2\text{CH}_2\text{COOH}$ . In order to be comparable the solutions were standardized to the same content of  $\text{NH}_2$  per

TABLE 1  
*A comparison of the sensitivity of the various procedures studied*

PROCEDURE	HIGHEST DILUTION WHICH GAVE A POSITIVE REACTION WITH:		
	Ammonium chloride	Methyl-ammonium chloride	Glycine
A	1-100,000*	1-100,000	1-10,000
B	1-500,000	1-10,000	1-5,000
C	1-10,000	1-5,000	1-5,000
D	1-1,000,000	1-50,000	1-100,000

\* Very faint reaction in 1-500,000.

5 cc. The following dilutions of  $\text{NH}_2$  were used: 1/100, 1/1,000, 1/5,000, 1/10,000, 1/500,000, 1/1,000,000.

In the case of the dilution of 1/100 the solution to be tested must have an alkaline reaction as the salts used in the tests shown above are acid in reaction and act as buffers. The glycine in all cases failed to give a reaction in strong solutions.

The procedure which uses a solution of phenol and the hypobromite (B) seemed to be the most promising according to these results. It was more sensitive towards ammonia than the Thomas test (A) and is not affected so much by methylamine. The original Thomas reaction (A) gave as pronounced a reaction with methylamine as with ammonia, although the color was not the same, being more greenish in the case of the former, and developed more slowly. In practice, however, it was difficult to distinguish

the two shades of color. The color in using the hypobromite solution (B) is not sky-blue like that of the Thomas reaction (A), but tends to a greenish blue, although it is just as easy to recognize. This particular practice (B), although applicable under certain conditions is unfortunately frequently difficult to handle. The color may disappear upon standing. It cannot be recommended unless further studies show it to be useful.

The substitution of the thymol for the phenol, but without substituting the hypochlorite by the hypobromite (C) has no especial advantage and has several disadvantages so that it may be dropped without further discussion.

The use of both the thymol and the hypobromite (D) is the method which seemed to be the best. It was more sensitive than the Thomas test towards ammonia, and less to methylamine. It is a little more sensitive than the Thomas test (A) towards glycine (the only disadvantage). This reaction is so extremely sensitive that even very small traces of ammonia may be detected. It may therefore be wise only to acknowledge distinct reactions as positive. Besides these advantages, it also has the advantage of eliminating the hypochlorite reagent as mentioned above.

All tests have to be applied in an alkaline solution or in a solution which is not more acid, than the alkaline reagent will neutralize. The well known Nessler's reagent must also be applied in an alkaline or neutral solution. The amount of alkali which must be added depends upon the acidity and the buffer action of the medium. For example, a 3.3 per cent aqueous solution of ammonium chloride (5 cc. were used for test) did not give any reaction with three (A) (C) (D) of the procedures, unless enough sodium hydroxide was added to give an alkaline reaction.

None of the nitrogen free organic compounds that have been tried gave any reaction with these reagents. The behavior of a primary amine has been described, and, of the amino-acids, glycine alone gave a distinct reaction. Alanine gave a faint reddish shade. Leucine, phenyl-alanine, tyrosine, asparaginic acid, asparagine, glutaminic acid, tryptophane, and cystine did not give any reaction. Neither did trimethylamine, aniline, pyridine, nor uric acid. A number of peptones were tried. In



some cases a reaction was obtained and upon further study certain brands of peptone were found not to be ammonia free.

When this test is to be applied in bacteriological technic, it is preferable to use agar slants as recommended by Hucker and Wall (1922). The organisms which are to be tested any be grown upon the following medium:

	per cent
Peptone.....	4.0
Glucose.....	0.2
Dipotassium phosphate.....	0.5
Agar.....	1.5
Water.	
Adjusted to pH 7.5.	

One of the great advantages of using agar slants for certain biological tests is that the growth products are collected in a small space, causing the reaction to be more pronounced as well as permitting short incubation. Another advantage in the use of agar slants is that the reagents are not directly mixed with the culture media. In this case, the reaction is more distinct and sensitive and produces the correct pH in the reagents without neutralizing the medium. After sufficient incubation the test is carried out as described above (p. 225). The agar is not mixed with the thymol solution although it may be desirable to mix the growth on the surface with the reagents.

A number of strains of bacteria have been tested for ammonia production from pepton using this agar and the thymol hypobromite test.

1 <i>Bacterium coli</i> Lehmann and Neumann	
1 <i>Bacterium fluorescens-liquefaciens</i> Flügge	
6 <i>Bacterium prodigiosum</i> Lehmann and Neumann	
2 <i>Tetracoccus liquefaciens</i> Orla-Jensen	
1 <i>Streptococcus liquefaciens</i> Orla-Jensen	
1 <i>Bacillus albolactis</i> Migula, No. 3	} American Type Culture Collection
1 <i>Bacillus cereus</i> Frankland, No. 21	
1 <i>Bacillus flavus</i> Ford, No. 58	
1 <i>Bacillus mesentericus</i> Trevisan, No. 76	
1 <i>Bacillus mycoides</i> Flügge, No. 80	
1 <i>Bacillus subtilis</i> Cohn, No. 102	
1 <i>Bacillus vulgatus</i> Trevisan, No. 123	

All of these strains gave a distinct reaction after incubation at their optimum temperature for 3 days.

This test may also be applied in a liquid medium of the formula given above, the agar being omitted. A longer incubation period is necessary in order to produce sufficient ammonia for the test. The medium is neutralized before adding the reagents. Thymol blue may be used as an indicator, and does not interfere with the reaction when ether is used. In an alkaline mixture this indicator is blue and this color is, of course, no indication of ammonia. Fortunately it cannot be shaken out with ether, so that an appearance of a red color in the ether is an indication of ammonia.

#### SUMMARY

A method for detecting ammonia, particularly to be used on agar slant cultures, is given. 1 cc. of a solution of thymol and 1 cc. of a hypobromite solution are added successively to the culture and allowed to act for 20 minutes. If ammonia is present the mixture becomes blue or greenish blue. The blue color may be extracted by means of ether in which it is soluble, resulting in a deep red-violet color. The reaction is also given by certain aliphatic amines and by glycine. It has the advantage over the Thomas test of not using a hypochlorite. The hypobromite is more easily prepared fresh and of definite strength each time it is used. This is done by mixing bromine water with sodium hydroxide. Like the Thomas test only weak solutions give the ammonia reaction.

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# THE RELATIONSHIP BETWEEN THE NITROGEN AND CARBON METABOLISM OF CLOSTRIDIUM ACETOBUTYLICUM<sup>1</sup>

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The relationship between the carbon and the nitrogen metabolism of a large number of organisms has been investigated by Kendall et al. (1913) (1922), Rettger et al. (1918a) (1918b) (1928), and Waksman (1917) (1925). For the most part these investigations deal with the specific problem of the sparing action of carbohydrate on the utilization of nitrogen by the various organisms. Little work is reported concerning the interrelationship between carbon and nitrogen metabolism. Wagner, Dozier, and Meyer (1924) report that the production of total volatile acids and the ratio of valeric : butyric : acetic acids was changed if glucose was added to cultures of *Cl. botulinum*.

In this study the effect of nitrogen metabolism on the carbohydrate metabolism was investigated, as part of the general problem of the utilization of various forms of nitrogen by *Cl. acetobutylicum*. This organism as reported by Peterson et al. (1924) (1926) has marked proteolytic activity in addition to its ability to ferment carbohydrates. The native proteins of corn, peas, rye, wheat, casein, etc. are rapidly hydrolyzed to soluble compounds. Protein split products, such as peptone, can also be used as a source of nitrogen.

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## EXPERIMENTAL

The culture employed, designated as A, was a strain of *Cl. acetobutylicum* used in the commercial production of butyl alcohol and acetone. Part one of this report deals with the fermentation of starch and various forms of nitrogen. These include two proteins—wheat gluten and casein, partially degraded proteins—Bacto-peptone, and completely degraded proteins, sold under the trade name of "aminoids."<sup>2</sup> The second part is concerned with the utilization of ammonia and nitrate nitrogen by the organism.

## ANALYTICAL METHODS

*Acidity.* For *total acid*, 10 cc. of the medium was diluted with 50 cc. of distilled water, heated to boiling, and after cooling, titrated electrometrically with 0.1 N NaOH to a pH of 6.7. It was found by adding known quantities of acid that the organic acids are estimated quantitatively at this pH and that free amino acids do not interfere. *Volatile acid* was determined by acidifying 100 cc. of medium with 1 N H<sub>2</sub>SO<sub>4</sub>, steam distilling, and titrating the distillate to phenolphthalein with 0.1 N Ba(OH)<sub>2</sub>. A portion (50 cc.) of the residue from the steam distillation was extracted with ether in a modified Soxhlet extractor and titrated for *non-volatile acid* with Ba(OH)<sub>2</sub> and phenolphthalein. The *H-ion* concentration was determined electrometrically with the quinhydrone electrode.

*Solvents and starch.* Total solvents (acetone, butyl alcohol, and ethyl alcohol), and the distribution of the individual solvents were determined by the methods previously reported (Fulton et al., 1926). Residual starch was hydrolyzed with HCl and then estimated by the micro-method of Stiles, Peterson, and Fred (1926).

*Forms of nitrogen.* Total nitrogen was determined by the standard Kjeldahl method. The fermented medium was filtered through paper pulp and *soluble* nitrogen determined in the filtrate by the same method. *Tungstic-acid-soluble* (non-protein)

<sup>2</sup> Arlington Chemical Company, Yonkers, New York.

nitrogen was estimated by the method of Hiller and Van Slyke (1922). Folin's aeration method was used for the determination of *ammonia* nitrogen. After removal of the latter, the analysis for *amino* nitrogen was made by Van Slyke's method. A modification of the Davisson-Parsons method (1919) was developed to determine *nitrate* nitrogen. One hundred cubic centimeters of the soluble nitrogen filtrate were made alkaline and evaporated to about 50 cc. Two cubic centimeters of basic lead acetate (33 per cent) and 6 cc. disodium phosphate (10 per cent) were added and the whole made up to 100 cc. To 50 cc. of the clear supernatant liquid, were added 150 cc. of water, sufficient 5 N NaOH to make the solution 0.1 N NaOH, and 2 grams of Devarda's alloy. This solution was slowly distilled into N/14 H<sub>2</sub>SO<sub>4</sub> until 100 cc. of distillate were collected. The excess acid was titrated with N/14 NaOH to the methyl red end-point. Blank determinations were made on the control flasks.

All the methods were carefully checked and found to give quantitative results when applied to the media used. The estimations of the various nitrogen fractions were accurate within 1 per cent, except for total nitrogen; in this case, due to inability to take representative samples, the duplicates varied from 1 to 2.5 per cent.

*Influence of form of nitrogen on acetone butyl alcohol fermentation.* Four experiments were made in which the nitrogen was supplied as protein, peptone, and amino acids (aminoids). The starch was special nitrogen-free starch obtained from the Corn Products Company, Argo, Illinois. In general, 2000 cc. of the medium were inoculated with 10 cc. of a twenty-four-hour culture of *Cl. acetobutylicum* in 7 per cent corn mash, incubated for three to five days at 37°C., made up to the original volume, and analyzed. During the fermentation, acidity and pH determinations were made periodically.

The data for the four experiments are given in tables 1 and 2, and in figures 1 and 2. The starch was not entirely fermented even after 136 hours. However, if the fermentation of carbohydrate was fairly complete, the distribution of the acetone, butyl alcohol, and ethyl alcohol, as evidenced by duplicate flasks did not appear to be influenced by the final yield.

TABLE 1  
*Action of Cl. acetobutylicum on simple and complex forms of nitrogen*

COMPOSITION OF MEDIUM	SOLUBLE NITROGEN									
	TOTAL NITROGEN		Total		Tungstic acid soluble		Amino		Ammonia	
	Begin-ning	End	Begin-ning	End	Begin-ning	End	Begin-ning	End	Begin-ning	End
	mgm. per liter	mgm. per liter	mgm. per liter	mgm. per liter	mgm. per liter	mgm. per liter	mgm. per liter	mgm. per liter	mgm. per liter	mgm. per liter
<b>Experiment I—72 hours:</b>										
Control, corn mash 7 per cent*	1,130	1,095	77	692	77	540	7	189	6	6
Starch plus beef peptone	1,130	1,095	1,130	1,040	744	744	188	266	22	4
Starch plus beef aminoïds	1,130		1,130	815	1,060	764	642	359	3	6
<b>Experiment II—72 hours:</b>										
Control, corn mash 7 per cent*	1,130	1,160	77	752	77	550	7	219		
Starch plus wheat gluten	1,130	1,135	112	688	72	627	9	119		
Starch plus beef peptone	1,130	1,140	1,130	1,032	744	755	188	308		
Starch plus beef aminoïds	1,130	1,130	1,130	870	1,060	770	642	441		
<b>Experiment III—96 hours:</b>										
Control, corn mash 7 per cent*	1,155	1,148	77	858	77	620	7	210		
Starch plus wheat gluten	1,140	1,140	112	820	72	812	9	170		
Starch plus beef peptone	1,155	1,156	1,155	1,100	750	812	190	338		
Starch plus beef aminoïds	1,155	1,208	1,155	976	1,084	848	653	463		
<b>Experiment IV—136 hours:</b>										
Control, corn mash 6 per cent*	970		66	784	66	620	6	217	5.5	13
Starch plus casein	970		40	788	40	575	10	194		8
Starch plus casein aminoïds	970	976	970	882	900	810	463	332		8

\* Dry corn.

*Discussion. Titratable acid and hydrogen ion.* As can be seen from figures 1 and 2 there are marked differences in the acidity and pH conditions of the various fermentations. The starch and

TABLE 2  
*Effect of form of nitrogen on carbohydrate metabolism*

COMPOSITION OF MEDIUM	STARCH			SOLVENTS			ACIDITY (1 N PER LITER)		
				Percentage of total solvents			Volatile cc.	Non-volatile cc	
	Beginning	End	Fermented	Total	Acetone	Ethyl alcohol			Butyl alcohol
Experiment I—72 hours:									
Control, corn mash, 7 per cent*				19.2				16.4 5.2	
Starch plus beef peptone . . . . .				16.7				19.9 3.2	
Starch plus beef aminoids . . . . .				16.4				17.2 4.8	
Experiment II—72 hours:									
Control, corn mash, 7 per cent*.	52.5	7.6	85.4	18.0	30.8	9.8	59.4	15.2 2.55	
Starch plus wheat gluten . . . . .	54.0	16.6	69.3	13.0	32.7	7.3	60.0	8.0 1.87	
Starch plus beef peptone . . . . .	54.0	18.1	66.5	11.5	34.3	5.2	60.5	18.8 2.75	
Starch plus beef aminoids . . . . .	54.0	13.1	75.8	14.2	31.1	6.2	62.7	15.9 3.38	
Experiment III—96 hours:									
Control, corn mash, 7 per cent*	52.5	5.2	90.1	19.4	30.0	10.7	59.3	18.4 4.75	
Starch plus wheat gluten . . . . .	54.0	8.0	85.2	17.0	30.1	11.0	58.9	24.4 2.50	
Starch plus beef peptone . . . . .	54.0	9.8	81.8	15.5	32.5	7.6	59.9	24.9 3.00	
Starch plus beef aminoids . . . . .	54.0	14.1	74.0	13.3	31.7	5.9	62.4	28.5 3.10	
Experiment IV—136 hours:									
Control, corn mash, 6 per cent*	45.0	4.0	91.2	16.6	30.2	10.4	59.4	20.8 2.38	
Starch plus casein . . . . .	45.0	2.7	94.0	14.2	32.0	8.3	59.7	18.2 3.87	
Starch plus casein aminoids . . . . .	45.0	4.0	91.2	15.8	34.0	6.1	59.9	25.2 2.20	

\* Dry basis.

protein (wheat gluten or casein) does not reach as high a peak as the corn control and this peak is several hours later; with casein this delayed maximum was especially noticeable. The pH values throughout most of the fermentation period are lower



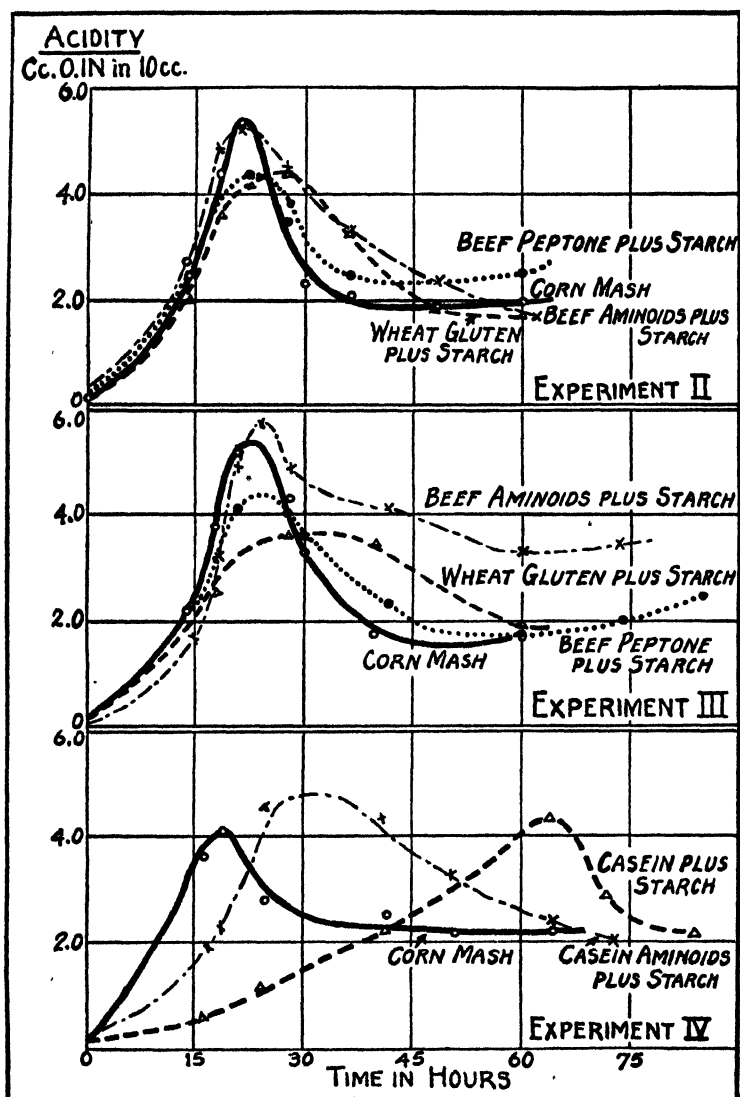


FIG. 1. EFFECT OF FORM OF NITROGEN ON THE TITRATABLE ACIDITY IN THE BUTYL ALCOHOL FERMENTATION

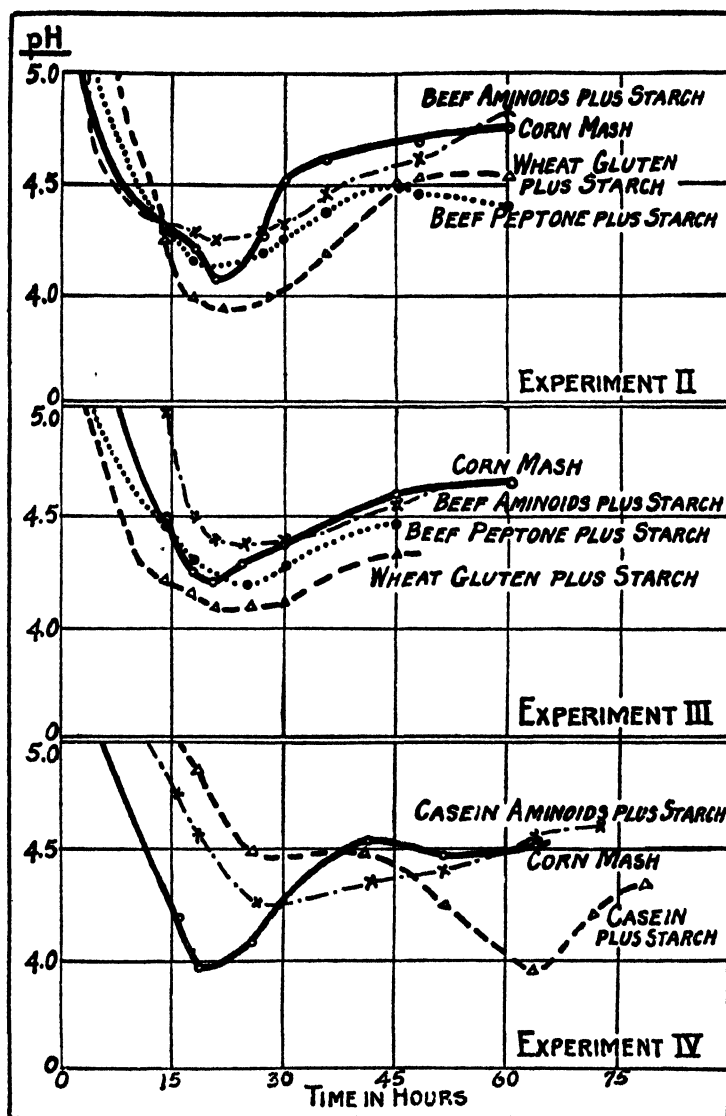


FIG. 2. EFFECT OF THE FORM OF NITROGEN ON THE pH OF THE MEDIUM

than those of the corn control; the final value is 0.2 to 0.3 less. In the case of the casein aminoids, the fermentation was delayed, hence the pH curve is displaced about forty hours from that of the corn control. These lower pH values are probably due to two factors, viz.: a smaller quantity of buffers from the decomposition of the protein and a higher concentration of acids after the peak in acidity has been passed. Starch and peptone gave acidity curves similar to those of the control, except that the peak was not so high. The fall in acidity was sharp, but toward the end of the fermentation a definite rise in acidity occurred. In spite of the degraded condition of the peptone the pH curves are in general lower than those of the corn control. This is especially true during the later stage of the fermentation. At this time, the acidity is higher in the peptone-medium than in the corn control. The acidity curves of the starch-aminoids fermentations are similar to those of the control except that the fall in acidity is not so abrupt. This delay is quite marked in the case of the casein-aminoids. The pH throughout the fermentation is much higher than in any of the other synthetic mixtures. It is especially noteworthy that after a rapid initial drop to 4.3 to 4.4 the pH rises rather slowly during the rest of the fermentation. This is probably due to the high buffering capacity of the aminoids as indicated by the large amount of amino nitrogen present.

*Nitrogen metabolism.* Three types of nitrogen metabolism are found in these experiments. The corn control and starch plus protein (wheat gluten and casein) represent a type wherein hydrolysis of complex forms predominates. There is a large increase in all of the soluble forms of nitrogen. Naturally, synthesis also takes place in the formation of cell protoplasm but decomposition of the protein covers the larger portion of the nitrogen changes. The second type is represented by the starch and peptone. In this case synthesis and decomposition are more nearly equal. Consequently the distribution of nitrogen at the beginning and end of the fermentation shows no pronounced changes. The total soluble fraction decreases 100 mgm. per liter and the amino increases by a like amount. In the starch and aminoids fermentation, synthesis of complex forms from

simple ones accounts for practically all of the changes in nitrogen. As a result, marked decreases (200 to 300 mgm. per liter) in soluble forms take place. The casein aminoids are not so completely degraded as the beef aminoids. This is shown by the smaller percentage of amino nitrogen in the casein product (table 1). On this account the synthesis of complex forms from amino acids is in part masked by the further hydrolysis of partially degraded forms already existent at the time of inoculation. An idea of the crop of the bacteria can be obtained from the decrease in soluble nitrogen. Analyses of *Cl. acetobutylicum* show that this organism contains 10.7 per cent nitrogen on a dry basis or 2.57 per cent on the wet. If it is assumed that all of the soluble nitrogen that disappears is converted into cells, 8 to 10 grams per liter of organisms should be formed in the starch-aminoids fermentation. Starch plus peptone should give a crop of 3.0 grams per liter. From bacterial counts and measurements it has been calculated that the average crop of bacteria in a corn fermentation is 2.0 to 3.0 grams per liter. Counts made by the Fries (1921) method on a starch-aminoid fermentation had a maximum count at twenty-four hours of  $4.00 \times 10^9$  bacteria per cubic centimeter as compared with  $2.54 \times 10^9$  per cubic centimeter for the corn control. After thirty-nine hours the former was  $3.74 \times 10^9$  per cubic centimeter while the latter had fallen to  $1.71 \times 10^9$  per cubic centimeter. Fermentations were made of aminoids plus 3 per cent glucose and the bacteria recovered at the end by means of a Sharples super-centrifuge. In one experiment 0.923 gram dry bacteria per liter was recovered as compared with 0.202 gram per liter for a peptone control. In a second experiment 0.452 gram per liter of dry bacteria was recovered from the aminoids-glucose fermentation and 0.154 gram per liter from the peptone-glucose. The nitrogen content of the bacteria which had grown on aminoids was 11.48 per cent as compared with 10.68 per cent for those grown on peptone. These figures indicate that there is a decided increase in cell protein in the presence of a more simple form of nitrogen, such as aminoids. Based on number of bacteria this increased cell protein was only double that in the corn control

whereas the nitrogen data of experiments I to IV indicated that there should be 3 or 4 times as many bacteria in the presence of aminoids. However, the total crop of bacteria may vary as much as 100 per cent as was shown by the experiments in which the bacteria were recovered. On the basis of the above data it cannot be definitely decided whether or not all of the soluble nitrogen that was converted into insoluble forms was built into cell substance. There is a large increase in cells in the presence of the aminoids but part of the nitrogen may be rendered insoluble during the fermentation by extra-cellular factors.

The question of the mechanism of the nitrogen utilization naturally arises. Previous work indicates that the first step is a splitting of the complex nitrogenous material to the amino acids. Schmidt, Peterson and Fred (1924), demonstrated the presence of leucic acid in a fermented corn medium and attributed it to the deaminization of leucine. Speakman (1926) cites experiments in which tyrosine was converted into p-hydroxyphenyl-lactic acid when added to the fermentation. He suggests that during the latter stages of the fermentation, after cell growth has ceased, the amino acids are deaminized and the ammonia thus formed stimulates the oxidation of the carbohydrate. The results of these investigators indicate that a portion of the amino nitrogen metabolized by the organism undergoes deaminization before it is used. However it is improbable that all or any considerable part of the amino acids is deaminized before they are utilized. A consideration of the nitrogen and acidity data of these experiments lends support to this view. An average of 200 mgm. per liter of alpha amino nitrogen disappeared during the fermentation of the starch and aminoids. This is equivalent to 143 cc. of 0.1 N acid. If we assume that this utilization was entirely intracellular, an equal quantity of non-volatile acid should be formed through deaminization. If the latter proceeded by oxidation or reduction the argument would still be valid, since only a few of the lower amino acids would yield volatile acids. The data show that 30 to 50 cc. per liter of non-volatile acid are obtained. Even if the entire quantity of these acids represent deaminization products they account for only 20 to 30 per cent

of the amino acids used. Therefore it seems probable that part of the amino acids are built directly into cells without deamination.

*Carbohydrate metabolism.* In spite of the differences in the nitrogen metabolism and accompanying changes in acidity and hydrogen-ion concentration, the distribution of the various products of carbohydrate decomposition was fairly constant. With beef peptone and casein-aminoids there was an increase in the acetone and a corresponding decrease in the ethyl alcohol. Beef aminoids on the other hand showed an increase in butanol at the expense of the ethyl. The differences in all cases were small although outside the limits of experimental error. It is likely that the difference noted is due to changes in the hydrogen-ion concentration and acidity of the fermentation caused by the type of nitrogen used. In general, the outstanding feature of the solvent distribution was the comparative constancy of the proportions of the products. Likewise the quantity of the acids at the end of the fermentation differed little and the differences were not constant among the several experiments. There appears to be a tendency for the fermentations in which the nitrogen was present as peptone or aminoids to have a slightly higher volatile acid content at the end. All fermentations showed a small content of non-volatile acid as compared to the quantity of volatile acid present. Variations in the amounts of non-volatile acid formed were independent of the source of nitrogen.

#### UTILIZATION OF AMMONIA NITROGEN BY *CL. ACETOBUTYLICUM*

Speakman (1926) and Weyer and Rettger (1927) have shown that *Cl. acetobutylicum* is not able to ferment glucose media if the sole source of nitrogen is an ammonium salt. However, the work on the production of hydroxy acids by Schmidt, Peterson and Fred, and by Speakman suggests that ammonia nitrogen can be used by the organism. The utilization of ammonia in the presence of organic nitrogen was investigated by the addition of various ammonium salts to corn medium. As a preliminary experiment, 7 per cent corn medium was prepared to which 25 to 100 mgm. per liter of ammonia nitrogen were added as chloride, phosphate, acetate, nitrate, or carbonate. Controls with the same

TABLE 3  
*Influence of ammonium phosphates on fermentation of corn mash*

	FLASK 1	FLASK 2	FLASK 3	FLASK 4	FLASK 5
Experiment I					
Nitrogen as ammonium phosphates, mgm. per liter.....	0*	24	47	98	200
Yield of solvents, percentage of dry corn..	27.1	26.9	27.3	26.5	16.7
Acetone, percentage of solvents.....	30.6	31.0	30.6	28.2	32.2
Nitrogen, mgm. per liter:					
Total:					
Beginning.....	1,190	1,204	1,237	1,285	1,390
End.....	1,190	1,207	1,226	1,280	1,420
Soluble:					
Beginning.....	77	101	124	172	277
End.....	875	912	920	860	376
Tungstic acid soluble:					
Beginning.....	77	101	124	172	277
End.....	616	630	632	560	244
Amino:					
Beginning.....	7	7	7	7	7
End.....	235	223	212	185	59
Ammonia:					
Beginning.....	6	30	53	104	206
End.....	9	12	9	6	5
Experiment II					
Nitrogen as ammonium phosphates, mgm. per liter.....	0*	46	69	92	138
Yield of solvents, percentage of dry corn	27.3	27.4	27.6	27.7	23.1
Acetone, percentage of solvents.....	30.5	28.7	25.9	25.9	30.8
Nitrogen, mgm. per liter:					
Total:					
Beginning.....	1,160	1,204	1,229	1,252	1,298
End.....	1,160	1,206	1,220	1,244	1,292
Soluble:					
Beginning.....	77	123	146	169	215
End.....	810	830	844	840	564
Tungstic acid soluble:					
Beginning.....	77	123	146	169	215
End.....	572	604	596	564	392
Amino:					
Beginning.....	7	7	7	7	7
End.....	196	201	202	187	105
Ammonia:					
Beginning.....	6	52	75	98	144
End.....	4	6	3	4	4

\* Control, 7 per cent corn mash plus 0.1 per cent  $\text{KH}_2\text{PO}_4$ .

quantity of these acid radicles in the form of the sodium salt were included and total solvents determinations were made after seventy-two hours incubation. The results showed that normal yields were obtained with 25 to 75 mgm. per liter of  $\text{NH}_3\text{-N}$ , but that the use of larger quantities resulted in a decreased yield.

TABLE 4  
*Effect of ammonium phosphate on fermentation of corn mash*

	FLASK 1—CORN MASH, 7 PER CENT*		FLASK 3—CORN MASH, 7 PER CENT PLUS 75 MG. PER LITER $\text{NH}_3\text{-N}$	
	Acidity†	pH	Acidity†	pH
Acidity at 15 hours.....	4.4	4.03	4.6	3.82
Acidity at 18 hours.....	3.8	4.12	3.8	3.97
Acidity at 21 hours.....	3.1	4.28	2.7	4.07
Acidity at 24 hours.....	1.6	4.45	2.0	4.16
Acidity at 39 hours.....	1.5	4.62	1.7	4.37
Acidity at 63 hours.....	1.8	4.62	1.9	4.50

*Solvents and forms of nitrogen at end of fermentation*

	FLASK 1*	FLASK 2*	FLASK 3	FLASK 4
Total solvents, gram per liter.....	18.22	19.38	18.48	19.26
Percentage acetone.....	33.5	31.0	31.5	28.3
Percentage ethyl alcohol.....	7.2	11.5	11.2	13.6
Percentage butyl alcohol.....	59.3	57.5	57.3	58.1
Total nitrogen, mgm. per liter.....		1,140		1,220
Soluble nitrogen, mgm. per liter.....	894	868	890	880
Tungstic acid soluble nitrogen, mgm. per liter.....	688	660	674	640
Amino nitrogen, mgm. per liter.....	238	228	220	220
Ammonia nitrogen, mgm. per liter.....	11.0	8.0	5.0	6.5

\* Plus 0.1 per cent  $\text{KH}_2\text{PO}_4$ .

† Cubic centimeters 0.1 N NaOH to bring 10 cc. of mash to pH 6.7.

*Effect of ammonium phosphate on fermentation of corn mash.*  
A number of flasks were prepared of 7 per cent corn medium in tap water, to which a mixture of equal amounts of the mono- and di-basic ammonium phosphate was added. To the controls was added 0.1 per cent of potassium acid phosphate. After seventy-two hours incubation at  $37^\circ\text{C}$ ., analyses for total solvents,



acetone, and forms of nitrogen were made. The data are given in table 3. In experiment II of this series analysis for ammonia-nitrogen was made immediately after sterilization and after sixteen hours. Quantitative recovery of the ammonia was obtained after sterilization which proved that none was lost through this process. After sixteen hours the flasks showed the following utilization of the ammonia nitrogen: 50 mgm. per liter, 95.0 per cent; 75 mgm. per liter, 95.5 per cent; 100 mgm. per liter, 76.0 per cent; 150 mgm. per liter, 55 per cent. These results show that a large proportion, though not all, of the ammonia nitrogen is used during the period of maximum growth and that the limit of ammonia utilization during this period is about 75 mgm. per liter. The results of the nitrogen distribution at the end show that in all cases the added ammonium nitrogen is completely utilized and there is no loss of nitrogen.

A third experiment was conducted in which nitrogen analyses were made periodically on an 8-liter sample to which 75 mgm. per liter of  $\text{NH}_3\text{-N}$  in the form of phosphates were added. The results are given in table 4 and in figure 3. The curves of nitrogen distribution show that during the early stages of the fermentation the presence of ammonium salts results in a decrease in the simpler forms of nitrogen. After 25 to 30 hours the flasks that contained the ammonium salt showed an increase in proteolysis so that the final nitrogen distribution in the control and ammonium phosphate flask showed no striking differences. Bacterial counts made by the Fries (1921) method gave the following data: fifteen hours, corn control  $1.94 \times 10^9$  bacteria per cubic centimeter, plus  $\text{NH}_3\text{-N}$   $2.87 \times 10^9$  bacteria per cubic centimeter; twenty-four hours, both contained  $3.3 \times 10^9$  bacteria per cubic centimeter.

The results of these three experiments demonstrate that  $\text{NH}_3\text{-N}$  as phosphate can be used by the organism up to 75 to 100 mgm. per liter without lowering the yield of solvents. Larger quantities reduce the yield, very likely due to liberation of the phosphoric acid. During the period of utilization of  $\text{NH}_3\text{-N}$  there is accelerated reproduction of the organism but the final crop of bacteria is not increased. From the slope of the curves in figure

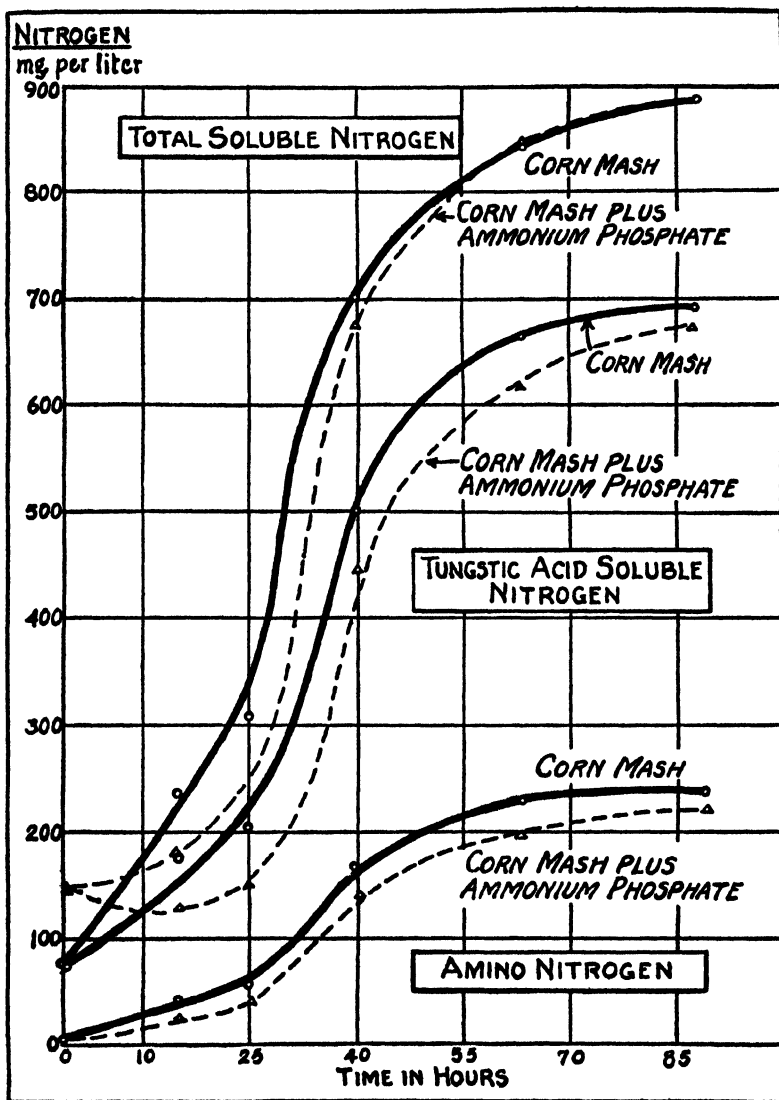


FIG. 3. EFFECT OF AMMONIUM PHOSPHATE ON THE NITROGEN METABOLISM OF CL. ACETOBUTYLICUM

3 it appears that as long as ammonia nitrogen is present there is a decrease in proteolysis but after the period of maximum growth is ended the rate of proteolysis is greater than that of the control so that the final nitrogen distribution does not differ markedly from that of the latter. The significance of this increase in rate of proteolysis is not readily apparent but might be an effect of change in reaction or an application of the law of mass action, i.e., proteolysis proceeds more rapidly after fifteen hours in the flask which had  $\text{NH}_3\text{-N}$  added on account of the smaller quantity of the end products of protein disintegration present. A reduction in proteolysis is indicated by the distribution of soluble nitrogen at the end of the fermentation. Since  $\text{NH}_3\text{-N}$  is built into cells and thus reduces the consumption of proteolytic products, the latter should be correspondingly increased if proteolysis is maintained. While, in general, there is more soluble organic nitrogen in the cultures to which  $\text{NH}_3\text{-N}$  was added than in the controls, the excess is not equal to the added  $\text{NH}_3\text{-N}$ . This reduction is still more evident from an examination of the tungstic-acid-soluble and amino nitrogen data. In the majority of cases the quantity of these substances is less in the fermentations to which ammonia was added than in the controls. These facts are strong evidence in support of the view that certain nitrogen requirements of the organism are met better by  $\text{NH}_3\text{-N}$  than by protein cleavage products. However, since the organism cannot subsist on  $\text{NH}_3\text{-N}$  alone, it appears that some amino acids as such are necessary for growth while others serve through deamination as a source of  $\text{NH}_3\text{-N}$ .

As can be seen from the data in tables 3 and 4 the presence of ammonium phosphate affects the production of solvents in two ways. If the amount of  $\text{NH}_3\text{-N}$  is less than 100 mgm. per liter, the yield of solvents is equal and often slightly greater than that of the control. However, the percentage of acetone is decreased, usually in proportion to the amount of ammonium phosphate added. There is a corresponding rise in the ethyl alcohol. If more than 100 mgm. per liter of  $\text{NH}_3\text{-N}$  is added, the yield is decreased and the percentage of acetone equals or exceeds that of the control, and the ethyl alcohol changes correspondingly.

The total acidity curve in the presence of ammonium phosphate differs only slightly from that of the control but the pH values are much lower throughout the fermentation. Therefore, it appears probable that a change in the hydrogen ion concentration is a contributing cause in altering the distribution of solvents.

*Effect of ammonium carbonate on fermentation of corn mash.* With ammonium phosphate the quantity of  $\text{NH}_3\text{-N}$  that can be

TABLE 5  
*Influence of ammonium carbonate on fermentation of corn mash*

	FLASK 1*	FLASK 2*	FLASK 3	FLASK 4
$\text{NH}_3\text{-N}$ added at:				
0 hours, mgm. per liter.....	0	0	47.1	47.1
13 hours, mgm. per liter.....	0	0	47.1	47.1
24 hours, mgm. per liter.....	0	0	47.1	47.1
31 hours, mgm. per liter.....	0	0	47.1	47.1
Total.....	0	0	188.4	188.4
Solvents, percentage of dry corn.....	26.6	26.5	25.8	25.5
Distribution of solvents:				
Acetone, per cent.....	32.3	32.3	31.8	31.8
Ethyl alcohol, per cent.....	8.3	8.8	8.0	7.5
Butyl alcohol, per cent.....	59.4	58.9	60.2	60.7
Nitrogen distribution at end, mgm. per liter:				
Total.....	1,114	1,140	1,360	1,340
Soluble .....	862	864	948	942
Tungstic acid soluble.....	628	624	712	732
Amino.....	218	223	237	237
Ammonia.....	11	9	25	29

\* Control.

used without lowering the yield is limited to about 75 to 100 mgm. per liter. This is due to the fact that with the utilization of the ammonia, phosphoric acid is liberated and this lowers the pH to such an extent that the fermentation does not proceed to completion. An experiment in which the ammonia was added as the carbonate was made in an effort to increase the amount of  $\text{NH}_3\text{-N}$  used by the organism without stopping the fermentation. The

acid liberated in this case is  $\text{CO}_2$  which should not be toxic; however, it is necessary to add the ammonium carbonate periodically in small quantities in order to avoid excessive alkalinity.

Six flasks containing 150 grams of corn and 1750 cc. of tap water were prepared; three served as controls. To the experimental flasks, sterile ammonium carbonate in dilute solution (2.5 mgm.  $\text{NH}_3\text{-N}$  per cubic centimeter) was added at intervals as shown in table 5. An equal quantity of sterile water was added to the con-

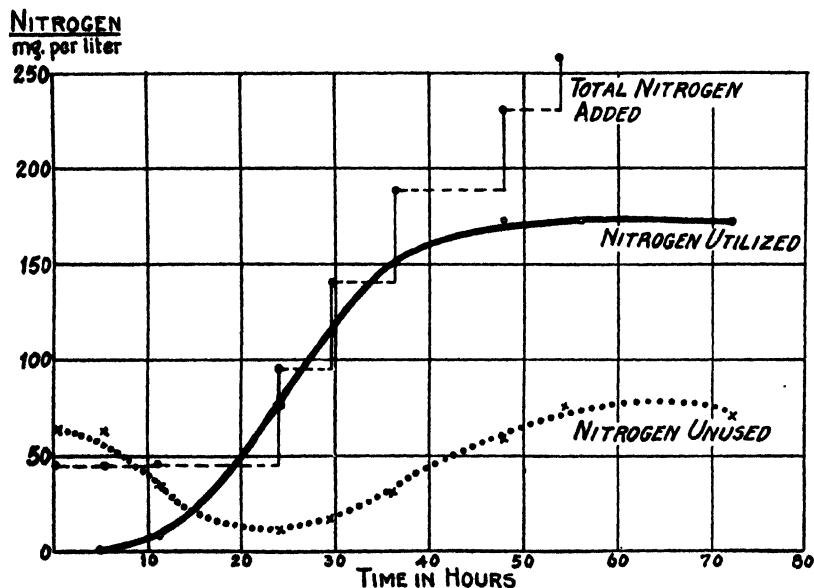


FIG. 4. UTILIZATION OF AMMONIA NITROGEN ( $(\text{NH}_4)_2\text{CO}_3$ ) BY *CL. ACETOBUTYLICUM*

controls. One control and one experimental flask were analyzed periodically in order to determine the rate of utilization of  $\text{NH}_3\text{-N}$ . At the end of thirty hours the addition of the carbonate to two of the experimental flasks was discontinued, but 150 mgm.  $\text{NH}_3\text{-N}$  as the carbonate was added in two portions to the third flask. Very little of this additional nitrogen was used as this flask contained 144.5 mgm. of  $\text{NH}_3\text{-N}$  at the end of the fermentation. The rate of utilization of the  $\text{NH}_3\text{-N}$  in the third experimental flask was determined and is shown in figure 4. The re-

sults of the analyses for solvents and nitrogen fractions at the end of the experiment are given in table 5.

The data show that the quantity of ammonium carbonate that can be used by the organism has a definite limit, about 175 mgm. per liter of  $\text{NH}_3\text{-N}$ . This nitrogen was used between the sixth and thirty-sixth hour. After the thirty-sixth hour the disappearance of ammonia was negligible. It is probable that the maximum utilization of  $\text{NH}_3\text{-N}$  was obtained in this experiment, since at no time was there a complete absence of this form of nitrogen.

The data on the nitrogen distribution (table 5) show that the addition of the carbonate increased the soluble forms of nitrogen. Evidently the  $\text{NH}_3\text{-N}$  is used by the organism in preference to the protein split products, and these accumulate. However, the increases in the soluble forms are not nearly enough to account for the ammonia nitrogen used by the organism. Counts made during the fermentation showed that the maximum number of bacteria was  $4.17 \times 10^6$  per cubic centimeter in the presence of ammonium carbonate as compared with  $2.54 \times 10^6$  bacteria per cubic centimeter for the control. These data indicate that part of this deficiency in the increases of the soluble forms is due to an increase in the crop of bacteria.

The presence of the ammonium carbonate does not appear to change the carbohydrate metabolism appreciably. The final yield is lowered slightly but the distribution of the solvent differs little from that of the control. The acidity and pH data (not given in table) are similar but addition of ammonium carbonate delays the time of maximum acidity about eight hours.

*Effect of various ammonium salts on solvent production.* In view of the results obtained with ammonium phosphate and ammonium carbonate a series of flasks were prepared in which the chloride and the sulfate were used in addition to the phosphate and the carbonate.

Thirty-seven and one-half grams of corn were made up with tap water and a solution of an ammonium salt added to a total volume 500 cc. and sterilized. The mash was inoculated with 10 cc. of a twenty-four-hour culture of *Cl. acetobutylicum*, incu-

bated seventy-two hours, and then analyzed for total solvents and solvent distribution. The carbonate was added as a sterile

TABLE 6  
*Effect of various ammonium salts on fermentation of corn mash*

COMPOSITION OF MEDIUM	ACID EQUIVA- LENT OF SALT ADDED	SOLVENTS			
		Total	Distribution		
			Acetone	Ethyl alcohol	Butyl alcohol
Series I					
	mgm. per liter	per cent dry corn	per cent	per cent	per cent
Control, 7 per cent corn mash .....	0	26.5	31.1	7.8	61.1
Phosphates:					
Plus 25 mgm. per liter NH <sub>3</sub> -N.....	131	26.0	30.5	8.8	60.7
Plus 50 mgm. per liter NH <sub>3</sub> -N.....	261	25.8	28.8	10.1	61.1
Plus 75 mgm. per liter NH <sub>3</sub> -N.....	392	26.3	26.7	13.7	59.6
Plus 100 mgm. per liter NH <sub>3</sub> -N.....	524	26.6	25.7	14.5	59.8
Chlorides:					
Plus 25 mgm. per liter NH <sub>3</sub> -N.....	65	26.1	30.5	8.0	61.5
Plus 50 mgm. per liter NH <sub>3</sub> -N.....	130	26.0	30.3	9.3	60.4
Plus 75 mgm. per liter NH <sub>3</sub> -N.....	195	26.4	29.1	10.8	60.1
Plus 100 mgm. per liter NH <sub>3</sub> -N.....	260	26.6	28.2	11.3	60.5
Series II					
Control, 7 per cent corn mash.....	0	26.6	31.1	9.1	59.8
Sulfates:					
Plus 25 mgm. per liter NH <sub>3</sub> -N.....	87.5	26.7	30.2	9.3	60.5
Plus 50 mgm. per liter NH <sub>3</sub> -N.....	175	26.6	30.0	9.3	60.6
Plus 75 mgm. per liter NH <sub>3</sub> -N.....	262	26.4	31.0	8.2	60.8
Plus 100 mgm. per liter NH <sub>3</sub> -N.....	350	25.0	33.1	6.8	60.1
Carbonates:					
Plus 21 mgm. per liter NH <sub>3</sub> -N.....		26.3	31.2	9.3	59.8
Plus 41 mgm. per liter NH <sub>3</sub> -N.....		26.3	30.8	7.8	61.4
Plus 52 mgm. per liter NH <sub>3</sub> -N.....		26.2	31.2	8.4	60.4
Plus 72 mgm. per liter NH <sub>3</sub> -N.....		26.6	30.6	9.2	60.2

solution after the sterilization of the corn mash. The results are given in tables 6 and 7. The values for the yield of solvents are the average of triplicate flasks all of which checked within 2.5 per cent. Residual ammonia determination on a large number of the flasks made at the end of the experiment showed that 95 to 100 per cent of the  $\text{NH}_3\text{-N}$  added had disappeared.

TABLE 7

*Effect of ammonium carbonate and sulfate on solvents, acidity, and pH*

COMPOSITION OF MEDIUM	FINAL ACIDITY*	FINAL pH	SOLVENTS			
			Total	Distribution		
				Acetone	Ethyl alcohol	Butyl alcohol
	cc.		per cent dry corn	per cent	per cent	per cent
<b>Sulfates:</b>						
Control, 7 per cent corn mash†	2 6	4.63	26.1	31.2	8.5	60.3
Plus 25 mgm. per liter $\text{NH}_3\text{-N}$	2 8	4.49	26.3	30.9	8.8	60.3
Plus 50 mgm. per liter $\text{NH}_3\text{-N}$	3 0	4.35	26.3	30.8	9.2	60.0
Plus 100 mgm. per liter $\text{NH}_3\text{-N}$	2 3	4.25	26.0	31.3	8.2	60.5
Plus 150 mgm. per liter $\text{NH}_3\text{-N}$	2 5	4.25	25.5	32.6	7.4	60.0
<b>Carbonates:</b>						
Control, 7 per cent corn mash	2 5	4.62	26.2	31.2	8.7	60.1
Plus 25 mgm. per liter $\text{NH}_3\text{-N}$	2 5	4.71	25.9	31.0	9.0	60.0
Plus 50 mgm. per liter $\text{NH}_3\text{-N}$	2 9	4.76	25.7	31.2	9.2	59.6
Plus 100 mgm. per liter $\text{NH}_3\text{-N}$	3 1	4.54	25.7	31.1	9.1	59.8
Plus 150 mgm. per liter $\text{NH}_3\text{-N}$	3 0	4.62	25.6	31.3	8.7	60.0
Plus 200 mgm. per liter $\text{NH}_3\text{-N}$	3.6	4.71	25.5	31.8	8.4	59.8

\* Cubic centimeter 0.1 N NaOH for 10 cc.

† Contained 0.1 per cent  $\text{Na}_2\text{SO}_4$ .

Results of this experiment reveal the fact that the action of the added ammonium salts cannot be explained solely by the effect of the liberated acid on hydrogen ion concentration. The phosphates and chlorides have much the same general effect, i.e., the acetone is lowered and the ethyl alcohol correspondingly raised. On the other hand fermentations in the presence of



ammonium sulfate showed much less change in the proportion of acetone. There was noticed a slight decrease with the lower concentration of the salt; as the amount added was increased the acetone content reached and exceeded that of the control. It is noteworthy that if the concentration of the salt added is increased to such a point that the yield of solvents is definitely lowered, an increase in the acetone content and a corresponding decrease in the ethyl alcohol results. This effect is independent of the salt added. Ammonium carbonate appears to have no effect on the solvent distribution in any of the concentrations used.

In consideration of these results, it appears that the change in the acetone and ethyl ratio in the presence of the ammonium salts is due to certain disturbances of the oxidative reactions that give rise to these compounds. These disturbances may be accomplished by changes in the hydrogen ion concentration especially as this affects the oxidation-reduction potential of the reactions. However, this alone does not seem to be sufficient, and it appears likely other factors are involved that are effective alone or in conjunction with the change in the pH. At present the identity of these factors is unknown but it is suggested that the specific effect of the acid radicle at the lowered pH may be important here. It has been shown in a previous publication (Wilson, 1927) that the addition of neutral or acid sodium phosphates to corn mash will result in a lower acetone content. The concentration of these salts of phosphoric acid necessary to effect this decrease is much higher than that used in our experiments but the effect of the phosphate radicle coupled with the decrease in pH might bring about the same change at lower concentration. Also, differences in the adsorption of the ions at the cell's surface might serve to explain the apparent anomalies offered by the various salts, especially the sulfate. Sulfuric acid in contrast to the other acids is negatively adsorbed in a solution of low concentration, i.e., raises the surface tension of water while the others lower it. Whether this difference would be great enough in the concentrations used to account for the effect of the ammonium sulfate is open to question.

Table 7 shows the effect on pH and final acidity of the ammonium salt of a strong acid, sulfuric, and a weak one, carbonic. With the former, the final acidity is changed little, but the pH is decreased in proportion to the quantity of salt added. With ammonium carbonate, the final acidity is higher than that of the control and the total solvents are slightly decreased, but the pH is practically unchanged. The solvent distribution is similar to those of corresponding flasks in previous experiments.

*Effect of inorganic acids on solvent production.* In the discussion of the results of the effect of ammonium salts on the fermentation it was assumed that the changes noted in the distribution of solvents were due to the acid liberated when the ammonia nitrogen of the added salt was used by the organism in its vital processes.

This assumption was tested by the addition to fermenting corn mash of the free acids previously used as the ammonium salts. Seven per cent corn mash was inoculated and after eighteen hours (i.e., at the peak of acidity) 0.1 N acids were added in quantities equivalent to those used as the salts of ammonia. This is not an exact duplication of the conditions of the experiments in which ammonium salts are used, since, in the latter case, the acids are liberated continuously from the start of the fermentation up to eighteen hours. However, results given in table 8 and figure 5 show effects qualitatively identical with those obtained when the acid was added as the ammonium salt.

If the acid added was less than the quantity equivalent to 100 mgm.  $\text{NH}_3\text{-N}$ , phosphoric and hydrochloric acid reduced the acetone content in approximately the same way as did the ammonium salts. The differences noted can also be observed in separate fermentations in which the same ammonium salt is used, e.g., the three fermentations with ammonium phosphate (Tables III and IV). If the acid content is increased beyond a certain limit, there is an abrupt drop in the yield of total solvents. With ammonium salts the drop was more gradual as the amount of salt was increased. This difference is perhaps due to the fact that the acid was added entirely at the peak, instead of gradually liberated as occurs when the ammonium salt is the

TABLE 8  
*Effect of inorganic acids on fermentation of corn mash*

FLASK	0.1 N ACID ADDED	NH <sub>3</sub> -N IN SALT EQUIVA- LENT TO ADDED ACID	SOLVENTS BASED ON DRY CORN	PERCENTAGE ACETONE IN SOLVENTS	FINAL ACIDITY*	FINAL pH
Series I—HCl						
	<i>cc. per liter</i>	<i>mgm. per liter</i>	<i>per cent</i>		<i>cc.</i>	
1†	0	0	{ 27.0 26.7	31.0 30.7	2.7	4.75
2	8.95	25	{ 26.8 26.4	29.9 29.7	2.7	4.66
3	17.9	50	{ 26.5 26.9	28.3 28.7	2.7	4.66
4	26.8	75	{ 27.15 27.15	28.8 28.2	2.4	4.59
5	35.7	100	{ 26.9 26.6	29.6 29.4	2.5	4.20
6	53.6	150	{ 6.2 4.8		4.7	3.35
Series II—H <sub>2</sub> SO <sub>4</sub>						
1†	0	0	{ 26.6 27.0	30.9 31.0	2.5	4.63
2	8.9	25	{ 25.9 26.8	30.2 30.6	2.7	4.46
3	17.8	50	{ 26.95 27.1	30.1 30.4	2.6	4.42
4	35.6	100	{ 25.1 25.1	32.3 31.9	3.5	4.03
5	53.4	150	{ 5.9 5.3		4.5	3.44
6	71.2	200	{ 5.4 5.7		4.6	2.93

TABLE 8—Concluded

FLASK	0.1 N ACID ADDED	NH <sub>3</sub> -N IN SALT EQUIVA- LENT TO ADDED ACID	SOLVENTS BASED ON DRY CORN	PERCENTAGE ACETONE IN SOLVENTS	FINAL ACIDITY*	FINAL pH
Series III—H <sub>3</sub> PO <sub>4</sub>						
1†	0	0	27.05	30.6	2.7	4.78
2	6.7	25	26.3	30.6	2.9	4.61
3	13.4	50	27.15 27.3	28.2 28.7	3.0	4.61
4	20.1	75	26.35 27.05	29.5 29.0	3.0	4.46
5	26.8	100	27.0 27.0	28.6 28.7	3.0	4.58
6	40.2	150	26.7 9.0	27.5	3.0 4.1	4.58 3.64

\* 0.1 N NaOH for 10 cc.

† Control, 7 per cent corn mash (dry basis).

source of the acid. Sulfuric acid gave a curve almost identical with that obtained with ammonium sulfate. The acidity and pH data show that the added acid produces little rise in the final acidity but causes a decrease in the pH.

Since this work was finished, Wynne (1929) in a preliminary report states that mineral acids appear to have no influence on the acetone production of this organism and that hydrogen-ion concentration *per se* has relatively little influence on the oxidative processes by which acetone is formed. Unfortunately, no data accompany this paper so that there is no way of ascertaining at this time the discrepancies between our results and his. The data presented in this paper show clearly that hydrochloric and phosphoric acids added to the fermentation in certain quantities will lower the acetone production without decreasing the total yield of solvents. Attention is called to the importance in this case of having a complete fermentation, since with lowered yields of total solvents the acetone content equaled and often exceeded

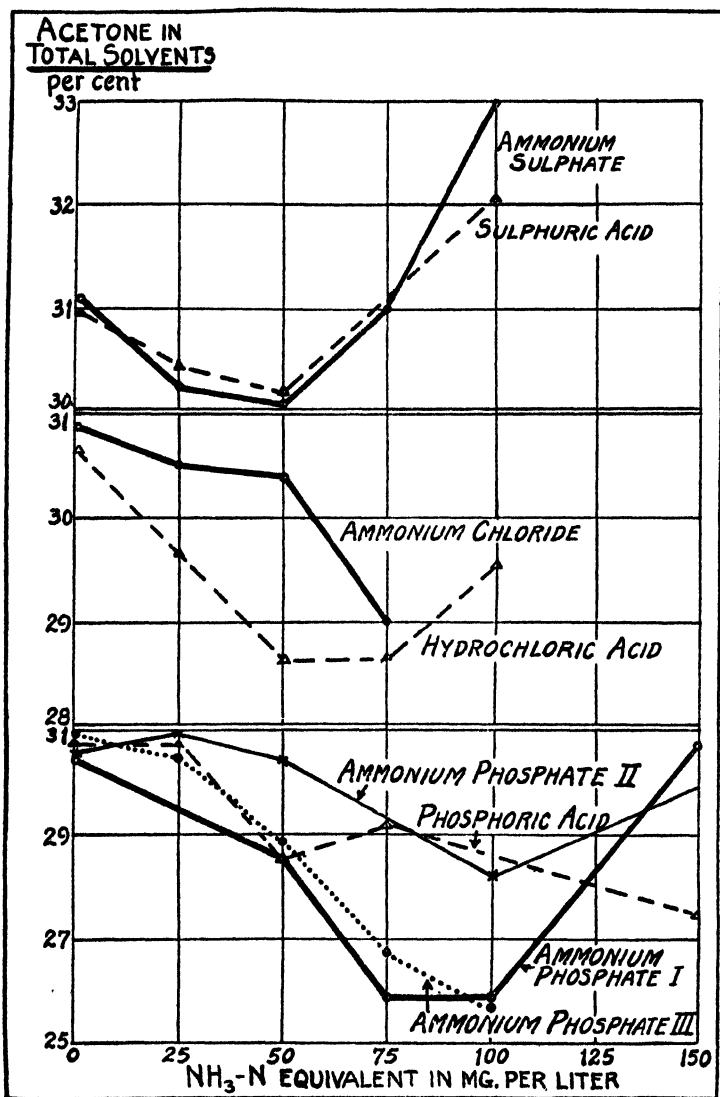


FIG. 5. EFFECT OF MINERAL ACIDS AND THEIR AMMONIUM SALTS ON THE ACETONE CONTENT OF THE SOLVENTS FROM THE FERMENTATION OF CORN

that of the control. Sulfuric acid appears to have little influence on the acetone content unless added in quantities sufficient to lower the yield, whereupon there occurs an increase in the proportion of acetone. The changes appear to be connected only in a general way with changes in the hydrogen-ion concentration.

*Utilization of ammonium salts in the presence of soluble forms of nitrogen.* As a result of the experiments in which the ammonium salts were added to corn medium, further experiments were made in which these salts were added to fermentations that contained a more simple form of protein. In the first experiment, ammonium carbonate was added periodically to a 3 per cent solution of glucose plus peptone. The nitrogen of the latter amounted to 1112 mgm. per liter, and 106.2 mgm. per liter  $\text{NH}_3\text{-N}$  was added. At the end of the fermentation 97.5 mgm. of the added  $\text{NH}_3\text{-N}$  was still present. In a second experiment the source of nitrogen was the soluble nitrogen from a previous fermentation of corn mash by *Cl. acetobutylicum*. After removal of the solvents, 25 grams per liter of glucose were added to the soluble nitrogen; this medium was sterilized and inoculated. The total nitrogen of this medium was 820 mgm. per liter. To one flask 101.0 mgm. per liter  $\text{NH}_3\text{-N}$  as the phosphate was added. After fermentation 98.0 mgm. of this was recovered. The third experiment was a duplication of the second except that the  $\text{NH}_3\text{-N}$  was added periodically as carbonate. Of the 147.0 mgm.  $\text{NH}_3\text{-N}$  added, 135.0 mgm. were recovered at the end of the fermentation. However, a fourth experiment in which peptone and beef aminoids were used with various ammonium salts, demonstrated that the organism can use appreciable quantities of  $\text{NH}_3\text{-N}$  in the presence of a degraded protein. The data given in table 9 show that the utilization of  $\text{NH}_3\text{-N}$  in the presence of peptone was fairly complete, but that, with aminoids, only about one-fourth of the added  $\text{NH}_3\text{-N}$  disappeared. However, if the amount of organic nitrogen present is decreased as was done in Series II there is a marked increase in the utilization of the  $\text{NH}_3\text{-N}$  in the presence of aminoids. Hence the organism can use  $\text{NH}_3\text{-N}$  in the presence of simple forms of organic nitrogen but the utilization does not occur so readily as in the presence of native

proteins. The fermentation is decidedly abnormal on the nitrogen-deficient medium as can be seen from the final yields of solvents and the percentage of acetone in the solvents. The effect of added ammonium salts on solvent production is essen-

TABLE 9

*Effect of various ammonium salts on fermentation of glucose and degraded proteins*

COMPOSITION OF MEDIUM	SOLVENTS		AMMONIA N	
	Total*	Acetone†	Beginning	End
Series I—organic nitrogen, 1130 mgm. per liter				
	<i>per cent</i>	<i>per cent</i>	<i>mgm. per liter</i>	<i>mgm. per liter</i>
Corn mash (7 per cent).....	26.4	29.2	6.0	10.0
Glucose (3 per cent) plus peptone.....	41.8	29.7	22.0	5.5
Glucose (3 per cent) plus peptone plus ammonium phosphate.....	41.1	27.2	114.4	9.9
Glucose (3 per cent) plus peptone plus ammonium chloride.....	36.7	29.0	115.5	18.1
Glucose (3 per cent) plus peptone plus ammonium sulfate.....	28.3	30.0	119.4	26.0
Glucose (3 per cent) plus aminoids.....	43.7	28.0	3.0	14.6
Glucose (3 per cent) plus aminoids plus ammonium phosphate.....	44.4	22.9	96.4	72.6
Glucose (3 per cent) plus aminoids plus ammonium chloride.....	34.3	24.8	95.4	67.0
Glucose (3 per cent) plus aminoids plus ammonium sulfate.....	42.7	25.5	100.4	74.0
Series II—organic nitrogen, 190 mgm. per liter				
Glucose (3 per cent) plus peptone.....	15.7	29.2	3.7	2.0
Glucose (3 per cent) plus peptone plus ammonium phosphate.....	14.5	29.8	96.1	13.4
Glucose (3 per cent) plus aminoids.....	22.0	25.8	0.5	2.0
Glucose (3 per cent) plus aminoids plus ammonium phosphate.....	31.0	25.6	93.8	25.0

\* Calculated on basis dry corn or glucose.

† Percentage of total solvents.

tially the same in these fermentations as that observed in the fermentation of corn. The fermentations required six days for completion and for this reason the percentage of acetone in all the flasks was slightly lower than usual because of the unavoidable loss of this solvent by evaporation.

*Effect of nitrates on the fermentation of corn mash.* It has been previously reported (Weyer and Rettger 1927) that nitrates cannot be used as the sole source of nitrogen by *Cl. acetobutylicum*. Two experiments were made to determine if nitrates are utilized in the presence of organic nitrogen. In the first, potassium nitrate equivalent to 100 to 200 mgm. N per liter was used. There was no utilization of the nitrate nitrogen and the distribution of the solvents was the same as that of the control. In the second experiment the nitrate was supplied as the ammonium salt (50 and 75 mgm. per liter  $\text{NO}_3\text{-N}$ ). The ammonium nitrogen was completely utilized but the  $\text{NO}_3\text{-N}$  was quantitatively recovered at the end of the experiment. The percentage of acetone was lowered as was observed with the ammonium phosphate and ammonium chloride. Tests for nitrites in both experiments were negative.

#### SUMMARY

*Cl. acetobutylicum* is able to use protein, peptone, or aminoids as sources of nitrogen with only slight changes in the ratio of solvents. If the source of nitrogen is peptone, there is a slight rise in acetone at the expense of the ethyl alcohol; with beef aminoids, a small increase in butyl alcohol is noted. There is evidence that the organism uses part of the amino acids as such, without deamination. In the presence of aminoids a large increase in synthesized protein takes place.

While the organism cannot use ammonia nitrogen alone, it appears to utilize ammonium salts preferentially in the presence of protein nitrogen. The maximum utilization of  $\text{NH}_3\text{-N}$  with complete fermentation was obtained with ammonium carbonate and amounted to 175 mgm. per liter. Ammonium salts of the mineral acids were utilized to a far less extent (75 to 100 mgm. per liter). Larger quantities, by reason of the liberated acid, prevent fermentation and thus decrease the yield of solvents. Ammonia nitrogen is used less readily in the presence of simple forms of organic nitrogen such as peptone and amino acids than in the presence of native proteins such as are found in corn.

The ratio of the solvents is changed if ammonium nitrogen is



supplied to the organism. In general, the effect is a decrease in acetone at the expense of the ethyl alcohol. If sufficient ammonium salt is added to cause a decrease in the total yield, there is a rise in the percentage of acetone. The effect of the presence of ammonium salts on solvent distribution is due to the acid liberated. The various acids apparently affect the solvent production in a general way by lowering the pH; there also appears to be a specific action of the acid.

Nitrate nitrogen, as the potassium or ammonium salt, is not used by *Cl. acetobutylicum* alone or in the presence of undegraded protein nitrogen.

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# THE REDUCTION OF NITRATES TO NITRITES BY *SALMONELLA PULLORUM* AND *SALMONELLA GALLINARUM*<sup>1</sup>

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The results of investigations dealing with the ability of *Salmonella pullorum* and *Salmonella gallinarum* to reduce nitrates to nitrites in a nitrate-peptone solution do not agree. Rettger and Harvey (1908) reported reduction by both organisms. Bushnell, Hinshaw and Payne (1926), Kaupp and Dearstyne (1927), Wallace and Neave (1927), and Tittsler (1928) reported reduction by *Sal. pullorum*. Hadley, Elkins and Caldwell (1918) found no reduction by either organism. Gage (1922), and Hadley, Caldwell, Elkins and Lambert (1917) reported no reduction by *Sal. pullorum*. Kaupp (1927) states that nitrates are reduced by *Sal. gallinarum* but not by *Sal. pullorum*. Bergey's Manual (1925) characterizes both organisms as negative to the nitrate test. Mallmann (1925) in an extensive study of *Sal. pullorum* found considerable variation. In 1924 his cultures gave a "weak positive test," but in 1925 some "were strongly positive, while others were weakly positive" and still others "were positive in only one tube." Perhaps the differences in results were due to differences in the media or methods of testing for nitrites; however, no definite conclusions can be drawn since no statements were made concerning these points except in the reports of Wallace and Neave (1927) and Tittsler (1928). The assumption that a "Standard Nitrate-Peptone Solution" was used would be erroneous, as evidenced by the report of Conn and Breed (1919) which

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is supported by an unpublished review of textbooks and laboratory manuals in bacteriology by the author. This review shows that the amounts of ingredients recommended vary greatly: peptone, from 0.1 to 1.0 per cent; potassium nitrate, from 0.02 to 1.0 per cent, while Chester (1901) specifies 0.002 per cent sodium nitrate; meat extract, although not included in most formulae, is specified in some as 0.3 per cent, and sodium chloride, used in only two formulae, as 0.5 per cent. Most formulae call simply for peptone, while three specify Witte's, one Parke-Davis and one Merck's. Similar variations are noted in the amounts of reagents employed in testing for nitrites. Some procedures call for only "a few drops" while others call for 2 cc. each of sulphanic acid and alpha-naphthylamine solutions.

Although the ability to reduce nitrates to nitrites is not employed in Bergey's (1925) classification to differentiate species within the genus *Salmonella*, it is used so extensively in other instances that consideration of these variations in media and methods of testing for nitrites is imperative. The investigation reported here was begun in 1927 to determine: first, if *Sal. pullorum* and *Sal. gallinarum* reduce nitrates to nitrites; second, to study variations between strains, and third, to study the adaptability of various nitrate-peptone media for this test.

During the progress of this study Wallace and Neave (1927) recommended the use of dimethyl-alpha-naphthylamine in place of alpha-naphthylamine in testing for the presence of nitrites; therefore, a comparative study was made of these two tests.

#### TECHNIQUE

##### *Cultures*

Two hundred and seventy-five strains of *Sal. pullorum* and ten strains of *Sal. gallinarum* were tested. The *Sal. pullorum* cultures came from widely separated sources and were of widely varying dates of isolation; 225 were isolated by the author over a period of five years and 50 were secured from eight different Agricultural Experiment Stations. Of the total number, 230 were isolated from chicks, 18 from septicemic infections in adult fowls, 15 from the ovaries of carriers, and 12 from eggs. Both the so-called

aerogenic and anaerogenic types were represented. The *Sal. gallinarum* cultures were also from various sources, nine having been secured from four Agricultural Experiment Stations, and one isolated by the author.

### *Media*

During the progress of this study many nitrate-peptone formulae were employed. In all cases, except those noted, Difco-peptone, C.P. nitrite-free potassium nitrate, Liebig's meat extract and distilled water were used. All media were sterilized at 15 pounds for twenty minutes.

### *Testing for nitrites*

Tests for nitrites were made after three to four days incubation at 37°C., using 1 cc. sulphanilic acid and 0.5 cc. of either alpha-naphthylamine or dimethyl-alpha-naphthylamine. The appearance of a red color with either test was considered to be indicative of nitrites.

### EXPERIMENTAL

One hundred and fifty strains of *Sal. pullorum* and nine strains of *Sal. gallinarum* were tested in 1927, using a nitrate-peptone solution made of Difco peptone 1.0 gram, potassium nitrate, 0.2 gram, and distilled water, 1 liter. Alpha-naphthylamine was used in testing for nitrites. All cultures reduced nitrates.

In 1928 these cultures and 50 additional strains of *Sal. pullorum* were tested under the same conditions, with similar results. Equally good results were obtained in a duplicate series of cultures where dimethyl-alpha-naphthylamine was used in place of alpha-naphthylamine.

In 1929 the same cultures, and also 75 strains of *Sal. pullorum* and one strain of *Sal. gallinarum* which had been isolated recently, were tested in standard nutrient broth plus 0.1 per cent potassium nitrate. Reduction of nitrate was obtained in every case. Therefore, both species were considered capable of reducing nitrates to nitrites in both of the above media. However, no explanation was at hand to account for the irregular results en-

countered by Mallman (1925) or the negative results of Gage (1922) and Hadley *et al* (1917, 1918). In view of the fact that so many formulae of nitrate-peptone solutions have been recommended, a study was made of various brands and amounts of

TABLE 1

*The effect of various kinds and amounts of peptone and nitrate upon the character of growth and reduction of nitrate by Sal. pullorum and Sal. gallinarum*

MEDIA				CHARACTER OF GROWTH	RESULTS	
Kind of peptone	Grams per Liter				Alpha-naphthylamine Test	Dimethyl-alpha-naphthylamine Test
	Pep- tone	KNO <sub>3</sub>	Meat extract			
Difco	1.0	0.2		+++	Good—very clear	Good—light
	1.0	1.0		+++	Good—very clear	Good—light
	1.0	2.0		+++	Good—clear	Good—clear
	1.0	5.0		+++	Good—very dark	Good—clear
	1.0	10.0		+++	Good—very dark	Good—clear
	0.5	1.0		+	Good—very clear	Poor—very light
	1.0	1.0		+++	Good—very clear	Good—light
	2.0	1.0		+++	Good—very dark	Good—clear
	4.0	1.0		++++	Very dark—precipitate	Very good—dark but clear
	8.0	1.0		++++	Very dark—precipitate	Very good—dark but clear
Proteose	1.0	1.0		++++	Good—very dark	Very good—clear
	1.0	1.0		+	Some negative—irregular	Poor—very irregular
Witte	2.0	1.0		++	Some negative—irregular	Poor—very irregular
	5.0	1.0		+++	Poor—irregular	Poor—irregular
	10.0	1.0		+++	Poor—irregular	Poor—irregular
Armours	5.0	1.0	3.0	+++	Good—clear	Good—light
	2.0	1.0		++++	Good—very dark	Very good—clear
Difco	5.0	1.0	3.0	++++	Negative or very strong—then faded	Very good—dark but clear
Difco		1.0	3.0	+++	Good—clear	Good light
	5.0		3.0	++++	Negative	Negative

peptone, various amounts of potassium nitrate, and of the influence of meat extract. In order not to make this portion of the study unnecessarily cumbersome, only ten strains of *Sal. pullorum* and three strains of *Sal. gallinarum* were used.

The formulae of media, types of growth and average results are summarized in table 1. These results show that both species reduced nitrate in a medium composed of from 0.5 to 8 grams of Difco peptone and from 0.2 to 10 grams of potassium nitrate per liter. It was noted that the greater the amount of peptone the greater was the amount of growth and the stronger the nitrite test. Both Difco proteose peptone and Armour's peptone gave equally good results. However, Witte's peptone not only gave poorer growth but either negative or irregular nitrite tests. A solution of 5 grams of Witte's peptone, 1 gram of potassium nitrate and 3 grams of Liebig's meat extract per liter gave approximately the same results as 1 gram of Difco peptone and 1 gram of potassium nitrate. These irregular results with Witte's peptone are in accord with the findings of Mallman (1925).

A nitrate-peptone solution composed of 10 grams of peptone and 0.02 gram of sodium nitrate per liter as recommended by Chester (1901) gave negative nitrite tests. However, good tests were obtained when 2 grams of sodium nitrate per liter were used. Perhaps the negative results obtained by a few investigators were due to the use of Chester's formula.

It was noted in the course of the study that 1 cc. of sulphanilic acid and 0.5 cc. of either alpha-naphthylamine or dimethyl-alpha-naphthylamine was sufficient to give a strong color; in fact only a few drops of alpha-naphthylamine were needed.

A comparison of the tests in which alpha-naphthylamine and dimethyl-alpha-naphthylamine were used favored the latter in that it always gave a clearer color, without any precipitate, while it never faded in solutions which contained meat extract.

#### DISCUSSION OF RESULTS

These results indicate that both the negative and irregular results of some investigators may have been due to either the use of Witte's peptone or of Chester's formula.

Although slight quantitative differences between strains were noted, they were not sufficient to be of importance except when Witte's peptone was used. Since some strains were negative and

others very irregular with this peptone, it is the author's opinion that it should not be used. The results obtained with various amounts of difco peptone show reduction of nitrate in various degrees. The amount of peptone which is desirable appears to be determined rather by the intensity of color desired in the nitrite test than by the qualitative results to be expected. The use of 2 grams of peptone and 0.2 gram of potassium nitrate per liter gave the most satisfactory results from the author's viewpoint. This was especially true when alpha-naphthylamine was used.

The use of standard nutrient broth plus one gram of potassium nitrate, as recommended by the Manual of Methods (1923), did not give satisfactory results with alpha-naphthylamine, as there was either an immediate fading or no appearance of color at all. However, when dimethyl-alpha-naphthylamine was used the color was permanent and satisfactory. The use of dimethyl-alpha-naphthylamine is highly recommended, especially to those who wish to use the standard broth-nitrate solution, because of the permanent color produced by it and the lack of appreciable precipitate. Attention is called to the slower development of color.

The results of a few tests showed that two days incubation at 37°C. were sufficient to give a strong nitrite test when 2 grams of Difco peptone were used.

#### CONCLUSIONS

1. Both *Sal. pullorum* and *Sal. gallinarum* reduce nitrates to nitrites.
2. No appreciable difference exists between strains.
3. Witte's peptone is not suitable for use in a nitrate-peptone solution.
4. A solution composed of 2 grams of Difco peptone, 0.2 gram of potassium nitrate and 1 liter of distilled water is satisfactory for these species.
5. Dimethyl-alpha-naphthylamine is superior to alpha-naphthylamine in testing for nitrites.

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# STUDIES OF BACTERIAL POPULATION DURING SLUDGE DIGESTION

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Bacterial population studies on digesting sludge mixtures have been carried out in the laboratory of the Sanitary District of Chicago during the past three years. The purpose of these studies was to determine the importance and relationship of the bacterial numbers and groups present to the rates of digestion. It was desired to compare the bacterial numbers present in various sludge mixtures digesting at different rates on the basis of the digestible substrate rather than on the volume of the liquor and also to determine the approximate bacterial rates of reduction of the digestible substrate.

## REVIEW OF BACTERIAL WORK ON SEWAGE SLUDGE

### *Fresh solids and Imhoff sludge*

The object of anaerobic sludge digestion is, briefly, to convert the putrescible sewage solids through the agency of bacterial activities to a relatively inert humus of reduced volume which can be easily air dried. The bacteriology of the digestion processes has not been studied so extensively as the chemical changes involved (Buswell, 1928). Many strains of bacteria have been isolated from sewage and their biochemical functions determined (Clark and Gage, 1905). However, the rôle and importance of the various biochemical groups in digestion is not well known. Correlation between any such groups and the course of digestion

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has yet to be definitely established. Winslow and Belcher (1904) studied the course of 15 groups of bacteria in sewage during storage on the basis of morphological and cultural characteristics. They found that obligate anaerobes were not abundant at any time, that facultative anaerobes predominated after forty-eight hours and that there was apparently no tendency toward any special forms. The groups found most representative were, cocci, chromogenes, *B. subtilis* group, *Bact. coli* group, *Bact. aerogenes* group and *Bact. rhinoscleromatis* group.

O'Shaughnessy (1914) compared the bacterial results from a well digesting sludge seven to eight weeks old with those from a poorly digesting foul smelling sludge three weeks old. The variations in the results from these sludges were largely in the 20°C. gelatin plate count, the 37°C. agar plate count, the *Bact. coli* group and the denitrifying group. There was little difference in the number of protein digesting, fat splitting and cellulose digesting organisms. Since the refractory sludge mentioned was derived from sewage containing trade wastes inimical to bacterial life, these results cannot be considered typical or significant. Gaub (1924) studied the organisms present in digested sludge at the time it was being drawn off for drying and reported that 80 per cent of the aerobes and 60 per cent of the anaerobes found belonged to the intestinal group, while 57.9 per cent of the aerobes and 40 per cent of the anaerobes liquefied gelatin.

Hotchkiss and Murray (1923) reported the numbers of proteolytic organisms, denitrifiers and nitrifiers found in the scum, liquid and sludge of an Imhoff tank. Hotchkiss (1924 and 1926) found no striking selective action in the types of bacteria which compose the flora of the Imhoff digestion chamber. She observed that nitrifying and sulfur oxidizing organisms are not eliminated in the digestion chamber. Variations in the operation of the tanks influenced the general bacterial flora and produced anomalous fluctuations which obscured seasonal effects. Hotchkiss (1926) also presents bacteriological data on the digestion of fresh solids under strictly anaerobic conditions and under semianaerobic conditions with the admission of small amounts of air during sampling. She concludes that the experiments give further

evidence of the succession of steps found in sewage digestion and also point to the importance of the oxygen supply in influencing the course of digestion. This last conclusion does not seem warranted without a thorough examination of the limits of variability in the performance of duplicate sludges under similar conditions and the precision of the bacterial indices used. Such an examination was apparently not made. Soppeland (1924) examined the top and bottom sludge in the digestion chambers of several tanks at the Calumet Treatment Works during the summer months. Very little numerical difference was *observed in the population* of the top and bottom sludge in the groups studied. The sludge at this plant had an average of 65 million acid formers in glucose broth, 280 million peptone digesters, 1.5 million gelatin liquefiers and 220 cellulose digesters per cubic centimeter.

The numbers of bacteria resulting in ripe sludge with the addition of various amounts of fresh solids daily have been determined by Rudolfs, Heukelekian, Zeller and Lackey (1926). They observed that after the addition of fresh solids a new equilibrium was reached which was above the control. The magnitude of the change in the equilibrium was independent of the amount of fresh solids added. The bacterial numbers were neither additive nor cumulative. The numbers of bacteria at any time within the bottles receiving various amounts of fresh solids seemed to depend upon the death rate of the intestinal organisms and the establishment of the normal flora of sewage digestion. The authors concluded that the addition of fresh solids beyond a certain maximum results in a retardation or derangement of the flora responsible for sewage digestion. High bacterial numbers did not seem to indicate rapid digestion. Excessive fluctuations apparently signified an unbalanced condition.

#### *Activated sludge*

Less bacteriological work has been done on activated sludge than on Imhoff sludge. Bartow and Russel (1915) isolated 13 strains of organisms from activated sludge. All of these were

aerobic except two which were facultative. Kamm (1916) found that denitrifying bacteria were always present in activated sludge and played an essential part in the purification. Courmont and Rochaix (1920) isolated seven strains of organisms from activated sludge. *B. subtilus* was one of these and the remainder were facultative anaerobes. Richards and Sawyer (1922) obtained variations from 4 to 17 million bacteria per cubic centimeter in activated sludge, depending upon the period of aeration. They state that a well aerated sludge contains about 1 million protozoa (flagellates, ciliates and sarcodinae) per cubic centimeter, and that there is positive correlation between the numbers of active protozoa and bacteria in activated sludge under varied conditions.

Buswell and Long (1923) describe activated sludge flocs as being composed of a synthetic gelatinous matrix similar to that of Nostoc or Merismopedeia, in which filamentous and unicellular bacteria are imbedded, and on which various protozoa crawl and feed. They noted that their results on the fauna of activated sludge were similar to those of Hommon (1918), but that they did not correspond to the results on sludge from larger experimental units. Reddie (1925) reported that in tanks in which grease destruction was taking place, liquefiers were present in abnormal proportion varying from 30 to 80 per cent of the total number of bacteria as compared to 10 to 20 per cent found in activated sludge under normal conditions. Greer (1925) examined activated sludge for anaerobes and reported from 10 to 10,000 *Cl. welchii* and equal numbers of *Cl. sporogenes* per gram of wet sludge. Harris (1926) states that the predominant organisms in Shieldhall sewage and activated sludge are similar and belong to the streptococci and "colon" groups. He also states that the sludge contains many other types of bacterial life, notably filamentous forms. Examination of the predominant protozoa showed that these organisms are subject to rapid changes in numbers and type and that these changes are dependent upon the system of aeration in operation, the season, and the strength and character of the sewage. Examinations of activated sludge made in this laboratory have shown variations in the

filamentous organism content of the *Sphaerotilus* type from 0 to 750 p.p.m. by volume. These studies all indicate that the flora and fauna of activated sludge apparently may vary materially due to various factors and still maintain the definite physical and biochemical characteristics of this sludge.

#### EXPERIMENTAL

##### *Digestion apparatus and sludge mixtures*

In each of our experiments the sludge for digestion was placed in a one-gallon Woulff bottle from which a gas outlet was provided leading to a 2-liter bottle filled with saturated salt solution. The setup was arranged so that the salt solution was automatically siphoned into a third bottle as gas was evolved in the digestion bottle. The Woulff bottle was arranged so that a sample of sludge could be drawn from the bottom while an equal amount of the same sludge, which had been stored in a separate bottle held at the same temperature, was added at the top.

Portions of each of the following sludge mixtures were put into duplicate sets of digestion bottles:

	MIXTURE 1	MIXTURE 2	MIXTURE 3	MIXTURE 4	MIXTURE 5	MIXTURE 6
Per cent activated .....	100	50	33	20	10	0
Per cent fresh.....	0	50	67	80	90	100

Percentage proportions refer to dry volatile solids. One series of these mixtures with gas collecting apparatus was incubated at 15°C. and will be referred to hereafter as Series A, while the other was incubated at 25°C. and will be called Series B. Samples for bacteriological and chemical analysis were taken twice a week during the period of active digestion.

##### *Methods*

The chemical examination of the sludge included determinations of organic solids, total solids, pH and analysis of the gas. The chemical determinations and results will not be discussed in this paper except in their application to the bacteriological

results. The bacteriological work included determinations of total plate colonies, glucose fermenters with acid and with gas production, gelatin liquefiers and anaerobic spores.

The total counts were made on glucose agar plates after an incubation period of forty-eight hours at 20°C.<sup>4</sup> In order to obtain as reliable counts as possible, four different dilutions with duplicate plates on each of the intermediate dilutions and single plates on the highest and lowest dilutions were planted on each sample. With this practice it was usually possible to count three or four check plates having between 25 and 400 well distributed colonies per plate.

Glucose fermenters with acid and gas production were determined in glucose broth fermentation tubes with Andrade's indicator, after the customary incubation period of forty-eight hours at 37°C. Gelatin liquefiers were determined by counting the number of liquefying colonies in gelatin tubes after an incubation period of forty-eight hours at 20°C.

In the estimation of the numbers of anaerobic spores of the *Cl. welchii* group the sludge sample was pasteurized at 80°C. for twenty minutes. Portions of the pasteurized sample were planted into milk tubes and into plates poured with a modified Wilson and Blair (1924) medium. The milk tubes were incubated at 37°C. for forty-eight hours and members of the *Cl. welchii* group were recorded as being present in the highest dilution showing stormy fermentation. The method of Wilson and Blair (1924) was followed in planting the iron (ferrie) sulphite agar medium but the amount of glucose was reduced from 1 to 0.1 per cent. This was done because 15 to 20 *Cl. welchii* colonies per plate usually blackened the plate to such an extent that it was difficult to count the number of colonies. It was found that if the amount of glucose was reduced, the spreading of the black areas could be checked without decreasing the productivity of black colonies. Even with the reduced amount of glucose in the medium the best results were obtained when the dilutions

<sup>4</sup> Later experiments showed that plain agar and caseinate agar are on the average productive of higher total counts from digesting sludges than glucose agar.

planted were such that between 25 and 100 colonies per plate resulted. With counts over 100, total blackening of the entire medium frequently occurred. Our practice with this medium was the same as for the glucose agar plates, that is, to plant four dilutions of each sample with duplicate plates for the intermediate dilutions. The iron sulphite agar plates were incubated at 37°C. for twenty-four hours. Greer (1926) stated that out of 160 black colonies picked from plates of this medium incubated at 37°C. one hundred and fifty-three were *Cl. welchii* and the remainder were *Cl. sporogenes*. We have, therefore, considered our counts on this medium as anaerobes of the *Cl. welchii* group without further investigation at this time.

#### *Treatment of data*

Twenty-one samples of each sludge mixture in Series B were examined at intervals up to one hundred and eighty days and 27 samples of each mixture in Series A were examined at intervals to two hundred and fifteen days. All results were expressed in numbers of bacteria per cubic centimeter of wet sludge. The reciprocal of the highest positive dilution was taken as the index for the organisms determined in the dilution tubes.

The method used for determining the trend curve for the groups determined in tubes and for the anaerobic spore counts was briefly as follows. The first sample in each series was taken as the initial. The next five samples were planted on the third, sixth, tenth, thirteenth and seventeenth days. The results on these days were averaged and were taken as a single point at the tenth day, the average age. In like manner points were determined for the thirtieth, sixty-first, ninety-first and one hundred and seventieth day for Series B. For Series A points were determined at the thirtieth, sixty-first, ninety-sixth, one hundred and fortieth and two hundred and fifteenth day.

The changes in the total counts were too great during the first ten or fifteen days to use the above method of determining a trend curve. Therefore, all points up to twenty days were plotted on semi-log paper and a smooth curve was drawn through them. After twenty days the same method was used as for the other



groups. The results obtained from the samples containing 80, 90 and 100 per cent fresh solids were very similar, with variations between them no greater than the probable error in the determinations. The results for these three mixtures were therefore

TABLE 1  
*Total plate count—average bacteria in millions*

AVERAGE AGE	SERIES A—DIGESTED AT 15°C.											
	Bacteria per cubic centimeter						Bacteria per gram organic solids					
	0	50	67	80	90	100	0	50	67	80	90	100
Percent fresh.	100	50	33	20	10	0	100	50	33	20	10	0
Per cent acti- vated .....												
<i>days</i>												
0	2.7	10		38			120	330		1,100		
3	5.2	24		130			250	700		3,300		
6	8.5	42		150			460	1,300		4,400		
10	14.0	55		160			800	1,900		4,400		
15	15.0	45		110			1,050	1,600		3,300		
30	6.9	15.5		45			400	570		1,400		
61	2.0	7.3		9.5			140	400		360		
96	2.6	6.3		11.0			160	310		500		
140	1.8	3.6		5.8			120	190		290		
215	1.3	3.7		3.7			95	200		190		
SERIES B—DIGESTED AT 25°C.												
0	4.0	10		36			220	360		910		
3	5.7	15		61			330	680		1,800		
6	7.4	20		83			430	890		2,300		
10	8.0	22		74			460	840		2,000		
15	6.2	17		45			320	590		1,200		
30	3.3	5.8		17			220	275		610		
61	1.1	2.4		4.9			87	135		250		
91	1.1	1.5		2.6			95	90		140		
170	0.6	0.24		0.21			37	15		12		

averaged and represent a mixture containing 80 per cent fresh solids and 10 per cent activated sludge. For the same reasons the two mixtures containing 33 and 50 per cent activated sludge were averaged. Our original data have therefore been condensed and will be presented to represent the following mixtures.

- 1—100 per cent activated sludge  
 2— 33 to 50 per cent activated sludge  
 3— 20 per cent or less activated sludge

The numbers of bacteria per gram of organic solids (digestible substrate) throughout the digestion period were calculated in the following manner. The number of grams of organic solids remaining were plotted against time and smooth curves drawn.

TABLE 2

*Acid formers, from glucose broth—average bacteria in millions per gram organic solids*

AVERAGE AGE	SERIES A—DIGESTED AT 15°C.					
Per cent fresh.....	0	50	67	80	90	100
Per cent activated.....	100	50	33	20	10	0
days						
0	55	34		28		
10	250	580		1,100		
30	48	34		45		
61	40	36		36		
96	30	13		9.3		
140	18	13		21.0		
215	7.0	0.52		2.0		
	SERIES B—DIGESTED AT 25°C.					
0	54	36		28		
10	37	170		380		
30	29	15		21		
61	63	31		45		
91	3.2	15		14		
170	5.0	7.2		2.4		

From these curves and the known volume the numbers of cubic centimeters of liquor per gram of organic solids were calculated. To illustrate, in Series A the 100 per cent activated sludge bottle contained 65.5 grams of organic solids in 3800 cc. of sludge liquor or 58 cc. of liquor contained 1 gram of sludge on the twentieth day. The number of bacteria per gram of organic solids was obtained by multiplying the number of bacteria per cubic centimeter by the number of cubic centimeters containing 1 gram of

TABLE 3

*Gelatin liquefiers—average bacteria in thousands per gram organic solids*

AVERAGE AGE		SERIES A—DIGESTED AT 15°C.					
Per cent fresh . . . . .		0	50	67	80	90	100
Per cent activated . . . . .		100	50	33	20	10	0
<i>days</i>							
0		16,500	18,500		150,000		
10		50,500	20,500		22,000		
30		29,500	2,500		2,200		
61		7,500	550		125		
96		1,700	250		275		
140		770	240		260		
215		350	—		210		
		SERIES B—DIGESTED AT 25°C.					
0		16,200	19,800		142,000		
10		32,000	3,900		12,500		
30		790	360		490		
61		730	460		320		
91		130	180		225		
170		590	290		435		

TABLE 4

*Anaerobic spores—average bacteria in thousands per gram organic solids*

AVERAGE AGE		SERIES A—DIGESTED AT 15°C.					
Per cent fresh . . . . .		0	50	67	80	90	100
Per cent activated . . . . .		100	50	33	20	10	0
<i>days</i>							
0		1,000	480		820		
10		2,100	1,900		2,300		
30		2,100	2,200		2,100		
61		1,300	1,400		1,900		
96		820	1,100		1,500		
140		630	820		1,400		
215		410	380		290		
		SERIES B—DIGESTED AT 25°C.					
0		1,000	560		1,100		
10		2,700	2,300		2,500		
30		2,400	2,400		3,000		
61		2,200	2,300		2,100		
91		1,900	1,800		2,500		
170		1,650	1,400		650		

organic solids. In this case 9,200,000 bacteria per cubic centimeter (total plate count) multiplied by 58 is equal to 533,600,000 bacteria per gram of organic solids on the twentieth day. The numbers of bacteria per gram of organic solids during the digestion period were treated the same as the numbers per cubic centimeter to produce trend curves for three representative sludge mixtures. Tables 1 to 5, inclusive, present the average bacteriological

TABLE 5

*Gas formers, from glucose broth—average bacteria in thousands per gram organic solids*

AVERAGE AGE	SERIES A—DIGESTED AT 15°C.					
Per cent fresh.....	0	50	67	80	90	0
Per cent activated.....	100	50	33	20	10	100
<i>days</i>						
0	5,500	1,800		20,000		
10	26,000	41,000		25,000		
30	4,800	1,200		450		
61	13,000	790		59		
96	2,800	120		13		
140	160	15		9.4		
215	7.0	5.3		3.5		
	SERIES B—DIGESTED AT 25°C.					
0	5,400	1,850		18,700		
10	13,500	5,750		4,900		
30	420	240		130		
61	46	180		43		
91	8.1	21		4.1		
170	9.2	33		6.0		

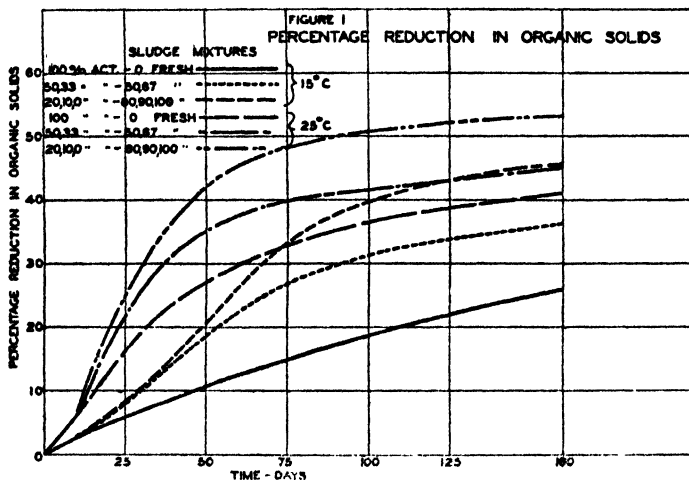
results obtained, as described, expressed in bacteria per cubic centimeter, and per gram organic solids.

### *Organic solids reduction*

The reduction in the organic solids obtained in these mixtures at the two temperatures is shown in figure 1. It is evident that the digestion process is much slower in the mixtures containing the greater percentages of activated sludge at each temperature and is very much slower at the lower temperature. It will also

be noted that 59 and 74 per cent of the total organic solids remained in the activated sludge alone after one hundred and sixty days at 25° and 15°C., respectively. In the mixtures containing little or no activated sludge only 47 and 54 per cent of the organic solids were left after one hundred and sixty days at these temperatures.

The changes in hydrogen ion concentration of these sludges during digestion were not great. The lowest pH (6.4) was observed in the sludges containing 20 per cent activated sludge or less. The greatest difference in the pH of all sludges was 0.4.

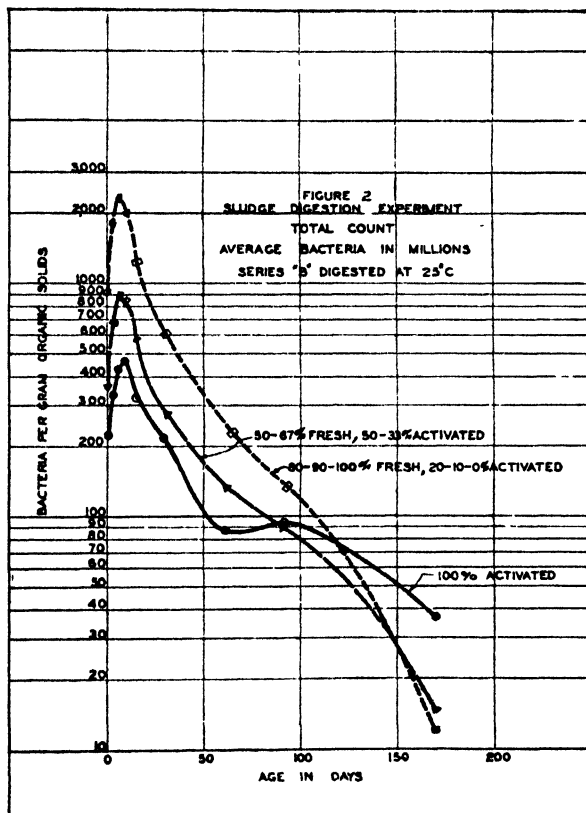


This occurred between 100 per cent activated sludge and 20 per cent or less activated sludge mixtures after sixty days at 15°C. At this time the pH values of these sludges were 6.8 and 7.2 respectively. It is not reasonable to assume that this slight difference in pH is significant in the retarded digestion of activated sludge at this temperature. Keeping in mind the difference in digestibility of these sludges as shown by the reduction in the organic solids, let us examine the bacteriological results obtained.

#### *Bacterial population trends*

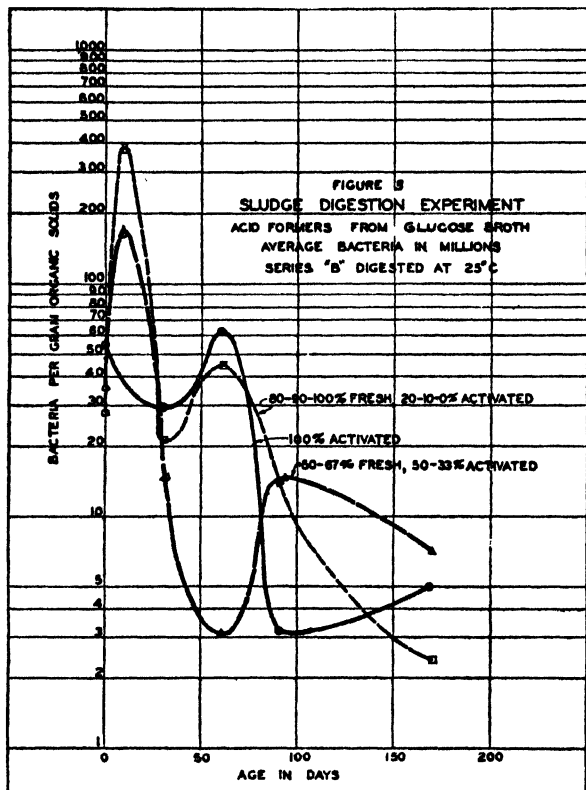
Since the curves obtained at 25° and 15°C. are of the same general type, only the 25° curves will be shown. The curve for

the total bacterial counts per gram of organic solids for 25°C. is shown in figure 2. The higher populations were obtained throughout most of the active digestion period in the mixtures containing the smallest percentages of activated sludge. At 25°C. the maximum population was reached in about six days



in mixtures of low percentage of activated sludge. As the percentage of activated sludge was increased the maximum point was retarded. This point was found at about ten days in activated sludge alone at 25°C. At 15°C. the maximum point was further retarded, not being reached until about the sixteenth day for activated sludge alone. The principal difference in the

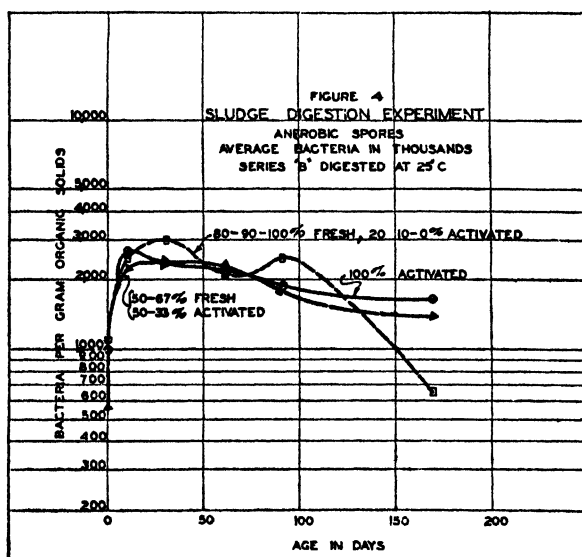
curves for the different temperatures occurred during the decline of the populations. At one hundred and seventy days 20 per cent or less, activated sludge mixtures had the smallest population. There was a tendency for the death rates to decline after the sixtieth day as the percentage of activated sludge was increased and also as the temperature was decreased.



In numbers, the glucose fermenters with acid production rank next to the total count. The curves for these organisms are shown in figure 3. As these are indices from dilution tubes in a geometric series the probable errors and variations incident to the method are greater than in the case of the total counts. The noticeable points in the 25°C. mixtures were the occurrence of a

second maximum at about sixty days and an initial decrease in numbers in the activated sludge sample in place of an increase to a maximum. At 15°C. the curves for the different mixtures were in general similar to the total count curves.

Perhaps the greatest difference in bacterial behavior in these sludge mixtures was shown by the gelatin liquefiers. (See table 3.) The numbers of gelatin liquefiers dropped rapidly in mixtures containing little or no activated sludge at both temperatures. In these mixtures the ratio of gelatin liquefiers to total count



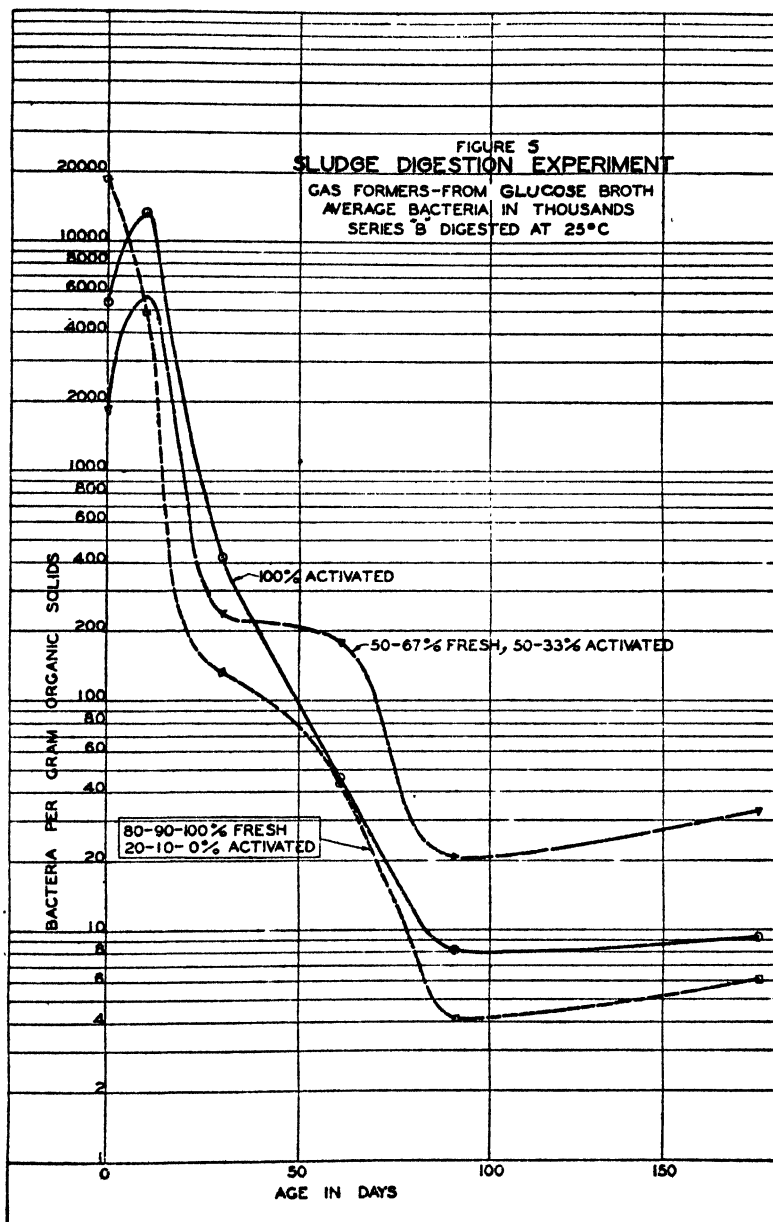
was reduced from 15 per cent at the start to less than 4 per cent at 25°C. and 0.25 per cent at 15°C. by the sixtieth day. The gelatin liquefying population of the mixtures containing 33 and 50 per cent activated sludge dropped rapidly from the start at 25°C. At 15°C. it rose to a maximum at ten days before the decline set in. In activated sludge alone there was an increase in this group at both temperatures before the decline. The rise was more pronounced and the death rates much lower at the lower temperature. This activated sludge also had the lowest reduction in volatile solids. Whether the apparent paucity of gelatin



liquefiers at the start, and their increase and slow death rate, are causative of, or merely incidental to, this retarded digestion is only conjectural at present.

The anaerobic spore plate counts as determined on the medium of Wilson and Blair are shown in figure 4. The anaerobic spores seldom represented more than 0.5 per cent of the total bacterial population. There was very little difference in the spore content in any of these sludges at the beginning of the experiment. There was no remarkable difference between the general trend of these curves for the various sludge mixtures. The maximum points were reached in from ten to twenty days but there was no rapid decline following, as for the other groups. In this case, the larger populations were found at the higher temperature. The milk fermentation tube results supported the plate counts but were more irregular and were not considered as reliable.

The curves for the glucose fermenters with gas production which include the *Bact. coli* and *Bact. aerogenes* groups are shown in figure 5. This group had a very high death rate from the start in the mixtures containing only small amounts of activated sludge at 25°C. This decline continued steadily until about the ninetieth day after which there was little change in the population level. By the ninety-first day there were only about 0.02 per cent of the group surviving in these mixtures. The mixtures containing 33 and 50 per cent activated sludge and the activated sludge alone at 25°C. had an increase in the glucose fermenters until the maximum was reached at about the tenth day. By the twentieth day the numbers in the group had fallen below the numbers of anaerobic spores in all mixtures at 25°C. and remained below them to the end of the experiment. The death rates for the glucose fermenters with gas production were much less at 15°C. than at 25°C. In the mixtures containing little activated sludge, there were rapid declines in this group from the tenth day until the ninety-sixth day. The other two curves at 15°C. showed lower death rates. The activated sludge contained large numbers of this group until the sixty-first day when a second maximum appeared but thereafter the decline was very rapid.



*Rates of bacterial reduction of organic solids*

The average rates of reduction of organic solids in per cent per day have been calculated. For this calculation the digestion period was divided into periods separated by the points for which the average bacterial counts were obtained. Each period was divided by the number of days in the period to obtain the average rate as shown in table 6.

It is evident that the maximum rates of organic solids reduction were obtained in the mixtures having 80 per cent or more fresh solids. The highest rates of reduction were in effect during the ten- to 30-day period for activated sludge and during the thirty- to sixty-one-day period for mixtures of fresh solids and activated

TABLE 6  
*Average percentage of organic solids reduced per day*

DIGESTION PERIOD	DIGESTED AT 15°C.						DIGESTED AT 25°C.					
	100 0	50 50	33 67	20 80	10 90	0 100	100 0	50 50	33 67	20 80	10 90	0 100
<i>days</i>												
0- 10	0.20	0.20		0.20			0.60	0.50		0.60		
10- 30	0.25	0.40		0.45			0.75	1.05		1.20		
30- 61	0.133	0.433		0.533			0.35	0.39		0.76		
61- 91	0.133	0.234		0.366			0.167	0.100		0.133		
91-160	0.130	0.087		0.116			0.087	0.058		0.044		

sludge at 15°C. At 25°C. the highest rate of reduction took place during the ten- to thirty-day period in all sludges.

We have no definite information as to the relative importance of any biochemical group of bacteria nor anything against the assumption that the total bacterial population is important. It will be interesting to examine the rates of digestion with reference to the total plate count. It can be asserted that all bacteria present in sludge play some part in the biochemical changes during their life cycle. It seems reasonable to assume that all groups obtained on the total plate count take some part, direct or indirect, in the organic solids reduction. We have therefore calculated the rate of organic solids reduction per billion bacteria

per day. To simplify the calculation it was based upon an initial amount of 100 grams of organic solids for each mixture. The average total number of bacteria present during a given digestion period was calculated from the average number of grams of organic solids remaining and the average total count during the period. The average total count during the period was determined by planimetering the proper bacterial curve. The rate of organic solids reduction in milligrams per day obtained from table 6 was then divided by the total number of bacteria present to obtain a rate expressed in milligrams of organic solids per billion bacteria per day. As an example, in the 100 per cent activated sludge at 25°C., during the thirty- to sixty-one-day

TABLE 7

*Average rate of organic solids reduction—milligrams per billion bacteria per day*

DIGESTION PERIOD	DIGESTED AT 15°C						DIGESTED AT 25°C.					
	100 0	50 50	33 67	20 80	10 90	0 100	100 0	50 50	33 67	20 80	10 90	0 100
<i>days</i>												
0- 10	4.8	1.8		0.61			17.2	6.9		3.2		
10- 30	3.4	3.6		1.8			30	25		14		
30- 61	5.9	11		9.8			32	32		31		
61- 91	10	8.9		13.5			26	15		14		
91-160	12	5.5		5.5			20	16		11		

period there was an average of 78 grams of organic solids remaining. There was an average bacterial content of 138 million per gram organic solids and a total of 78 by 0.138 or 10.8 billion bacteria. The average rate of organic solids reduction during this time was 350 mgm. per day. This is equal to 350 divided by 10.8 or 32 mgm. per day per billion bacteria. The rates obtained by this method are shown in table 7.

To simplify the comparison of the bacterial rates of digestion per billion bacteria a weighted average for various periods of digestion was calculated from table 7. For this calculation each ten-day period was given a weight of one and the average rates of reduction of organic solids per day per billion bacteria

obtained are shown in table 8. Whether the bacterial rates of organic solids reduction that are shown in tables 7 and 8 are common or efficient ones we have no way of telling at the present time. It is probable that such rates will vary considerably in sludges collected at different seasons or from different places. These rates are presented at this time not as a standard of performance but simply to illustrate the possibilities of such a method of analysis.

An examination of table 7 shows a greater variation in the rates at the various periods for the mixtures containing the higher amounts of fresh solids. At 15°C. there is not much difference in the rates per billion bacteria for the different sludge mixtures

TABLE 8

*Weighted average rate of organic solids reduction—milligrams organic solids per billion bacteria per day*

DIGESTION PERIOD	DIGESTED AT 15°C.						DIGESTED AT 25°C.					
Percent activated .....	100	50	33	20	10	0	100	50	33	20	10	0
Percent fresh	0	50	67	80	90	100	0	50	67	80	90	100
days												
30	3.8	3.0		1.4			25	19		10		
60	4.9	7.2		5.6			29	25		21		
100	7.3	7.6		8.0			27	21		18		
160	9.1	6.8		7.0			24	20		15		

after the first ten days. The rates for the activated sludge are slightly higher than for the sludges containing the higher amounts of fresh solids during all periods at 25°C. The natural conclusion from this is that the flora present in the activated sludge was accomplishing as much in the way of organic solids reduction per billion bacteria as the flora in the fresh solids at 25°C. Hence, from a biological standpoint, it would seem unnecessary to seed this activated sludge with fresh solids to obtain a better flora for digestion. It may be argued that the large total population in fresh solids is not significant but that the organic solids reduction is brought about by certain definite groups of smaller total numbers. Such possible groups have yet to be isolated. It also

appears significant that all biochemical groups that have been studied in fresh solids are also found in large numbers in activated sludge. Bacterial seeding with small amounts of fresh solids will not stimulate the growth of organisms that are already present in activated sludge. We have calculated the theoretical number of bacteria which would be present by mixing this digesting activated sludge with the digesting fresh solids at any one time. These were compared with the actual numbers obtained in the

TABLE 9

*Comparison of the actual and theoretical numbers of bacteria for sludge mixtures—total plate count—millions of bacteria per gram organic solids*

AGE	50 PER CENT ACTIVATED, 50 PER CENT FRESH				67 PER CENT FRESH, 33 PER CENT ACTIVATED			
	Digested at 15°C.		Digested at 25°C.		Digested at 15°C.		Digested at 25°C.	
	Number actually found mixed at start	Theoretical number if mixed at indicated age	Number actually found mixed at start	Theoretical number if mixed at indicated age	Number actually found mixed at start	Theoretical number if mixed at indicated age	Number actually found mixed at start	Theoretical number if mixed at indicated age
<i>days</i>								
0	340	810	360	810	350	1,000	360	870
3	600	2,200	750	1,300	800	2,900	600	1,600
6	1,050	3,100	960	1,600	1,450	3,900	810	1,900
10	1,700	3,100	840	1,400	2,050	3,900	840	1,700
15	1,800	2,600	550	860	1,500	3,000	620	1,000
31	530	950	260	500	600	1,120	290	590
61	370	300	170	180	430	360	100	219
96	340	380	90	150	270	450	85	165
140	220	280			160	330		
170			18	30			12	18
215	170	137			215	150		

sludge mixed at the start (table 9). This showed that the initial mixing did not produce trends to the higher population levels of fresh sludge but in almost every case did trend toward the lower population levels of activated sludge. In other words mixing in this case did not stimulate total bacterial growth to higher levels.

To return to the rates of digestion, the data indicated that throughout the practical digestion period the rate per billion bacteria was slightly higher for the digestion flora established by

activated sludge in its menstrum than for the flora in mixtures containing 80 per cent or more fresh solids at 25°C. At 15°C. there was no appreciable difference in the efficiency of the digestion flora of these sludges. This suggests that the flora arising in activated sludge during digestion may be an efficient one in reducing organic solids. We have no data to show that this is generally true. Our data do show that even in this case the activated sludge digested slower than fresh solids. As shown by figure 1 the mixtures containing 80 per cent or more fresh solids digested faster and to a greater extent. But the rate of bacterial reduction of organic solids in activated sludge was greater at 25°C. and this bacterial rate had less change throughout the

TABLE 10

*Ratios of the weighted average rates of reduction of organic solids per day per billion bacteria for 15°C. and 25°C.*

PER CENT ACTI- VATED ..... PER CENT FRESH .....	100		50 33		20 10 0		
	0		50 67		80 90 100		
Average for days	15°C.	25°C.	15°C.	25°C.	15°C.	25°C.	
30	1.0	6.5	1.0	6.4	1.0	7.2	
60	1.0	5.9	1.0	3.5	1.0	3.7	
100	1.0	3.8	1.0	2.8	1.0	2.2	
160	1.0	2.6	1.0	2.9	1.0	2.2	

course of digestion than the corresponding rate for fresh solids. In our opinion this indicated a difference in the digestibility of these sludges which is due to inherent characteristics other than bacterial flora. Compared to fresh solids, on the basis of its bacterial performance in reducing organic solids, this activated sludge apparently did contain the proper digestion flora. It may be possible to change the characteristics of activated sludge so that a greater proportion of its organic solids content will be digested in a given time.

The ratios of the weighted average rates of organic solids reduction per day per billion bacteria at the two temperatures have been calculated and are shown in table 10. The 10°C. rise

in temperature increases the bacterial rate of organic solids reduction between 6.4 and 7.2 times for the first thirty days. The average rate for sixty days, however, was 3.7 times for 20

TABLE 11

*Comparison of bacterial flora during the period of maximum organic solids reduction per billion bacteria*

All bacterial counts expressed in thousands per gram organic solids

PER CENT ACTIVATED .....	100 0		50 50	33 67	20 80	10 90	0 100
PER CENT FRESH .....							
Sludge mixtures	Digested at 15°C.	Digested at 25°C.	Digested at 15°C.	Digested at 25°C.	Digested at 15°C.	Digested at 25°C.	
Period of maximum rate of organic solid reduction, days....	91-160	30-61	30-61	30-61	61-91	30-61	
Milligrams solids per day per billion bacteria.....	12.25	32.4	11.50	31.8	13.5	31.4	
Total count.....	140,000	138,000	485,000	180,000	430,000	390,000	
Acid formers from glucose broth..	24,000	41,000	35,000	23,000	23,000	33,000	
Gelatin liquifiers.....	1,200	760	1,500	410	200	405	
Anaerobic spores.....	725	2,300	1,800	2,350	1,700	2,550	
Gas formers from glucose broth...	1,480	230	945	260	36	86	
pH.....	7.1	7.2	7.1	7.4	7.3	7.4	

TABLE 12

*Group count percentages of total count during the period of maximum organic solids reduction per billion bacteria*

PER CENT ACTIVATED .....	100 0		50 50	33 67	20 80	10 90	0 100
PER CENT FRESH.....							
Sludge mixtures	Digested at 15°C.	Digested at 25°C.	Digested at 15°C.	Digested at 25°C.	Digested at 15°C.	Digested at 25°C.	
Acid formers from glucose broth..	17.1	29.7	7.2	12.7	5.35	8.5	
Gelatin liquifiers.....	0.86	0.55	0.31	0.23	0.04	0.10	
Anaerobic spores.....	0.52	1.67	0.39	1.30	0.39	0.65	
Gas formers from glucose broth..	1.06	0.17	0.19	0.14	0.008	0.022	
Total of above groups.....	19.54	32.09	8.09	14.37	5.788	9.27	

per cent or less activated sludge mixtures and 5.9 times for 100 per cent activated sludge. The average for one hundred days was also accelerated to a greater extent in the activated sludge alone by this increase in temperature.



It is logical to assume that the optimum flora for digestion will obtain when the rate of reduction of organic solids per billion bacteria is greatest. It will be interesting, to compare the numbers of the groups studied that were present during these periods in the various sludges. This comparison has been made in table 11 and shows that all of these maximum rates occurred when the average total plate counts were between 138 and 485 million bacteria per gram organic solids. The percentage of the total count represented by each group of bacteria at the time of the maximum rate of bacterial reduction of organic solids has been calculated from table 11 and is shown in table 12. An examination of this table shows that there is a similarity in the percentages of each group present in a given sludge at the two temperatures. In all cases the total of these groups is less than 35 per cent of the total count and in three cases less than 10 per cent. These groups make up a larger percentage of the total count in activated sludge alone than in the mixtures containing the larger amounts of fresh solids. The total percentage of these groups in each mixture was always greater at 25°C. than at 15°C. It may be concluded that in the groups examined no significant combination appears which is likely to be present during periods of rapid digestion.

#### CONCLUSIONS

Six sludges, including activated sludge alone, fresh solids alone and four different mixtures of the two, were allowed to digest at 15° and 25°C. The reduction of organic solids, pH changes and changes in the bacterial population were followed during the course of digestion.

The greatest reduction of organic solids was found in the mixtures containing 20 per cent activated sludge or less (on the dry organic solids basis) and the least in the activated sludge alone. The digestion was much slower at 15°C. than at 25°C. After one hundred and sixty days the reduction in organic solids was 26 and 41 per cent at 15° and 25°C., respectively, for activated sludge alone; and 46 and 53 per cent at these temperatures for the mixtures containing 20 per cent or less of activated sludge with 80 per cent or more fresh solids.

The pH changes during the digestion of these sludges were not great. The mixtures containing 80 per cent or more of fresh solids had the lowest pH at the start, 6.4, but the pH increased steadily until 7.5 was reached. The activated sludge alone started at 7.3 and fell to 6.7 in ten days after which it slowly increased to 7.2 at 15°C. and 7.4 at 25°C. The difference in the pH of these sludges is not considered responsible for the retarded digestion of the activated sludge at the lower temperature.

Trend curves for the population changes in the total plate counts, acid and gas formers from glucose broth, gelatin liquefiers and anaerobic spores in the several sludges were derived. In general, the populations increased for about ten or fifteen days until an equilibrium was reached after which rapid declines set in and continued throughout the practical digestion period. The trend curve for the gelatin liquefiers in the mixtures containing 80 per cent or more of fresh solids is an exception, in which the decline began at once. The numbers of anaerobic spores underwent the least change after the equilibrium point was reached and were of similar magnitude in all sludge mixtures. The total bacterial populations were, as a rule, highest in the mixtures having the most fresh solids and lowest in the activated sludge alone.

It was assumed that the total bacterial population was responsible for sludge digestion and the rates of organic solids reduction per day per billion bacteria (total plate count) were calculated. These rates were highest in activated sludge alone at 25°C. This indicated, if the above assumption is correct, that the flora present in this activated sludge was apparently as efficient in organic solids reduction as the flora of fresh solids.

A comparison of the rates of organic solids reduction per billion bacteria showed that the average rate for one hundred days was 2.2 times as great at 25°C. as at 15°C. for mixtures containing 20 per cent activated sludge or less. For activated sludge alone, this rate was 3.8 times greater at the higher temperature.

The maximum bacterial rates of organic solids reduction

occurred in all sludge mixtures when the total plate counts were between 138 and 485 million bacteria per gram organic solids. An examination of the ratios of the groups present in the sludge mixtures during the period of maximum organic solids reduction per billion bacteria failed to show any significant relationships.

In conclusion we wish to acknowledge the helpful suggestions and criticism of F. W. Mohlman under whose direction this work was carried out.

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# GERMICIDAL EFFICIENCY OF CHLORINE AND THE N-CHLORO DERIVATIVES OF AMMONIA, METHYLAMINE AND GLYCINE AGAINST ANTHRAX SPORES

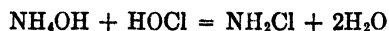
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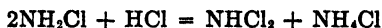
Effective disinfection of tannery effluents against anthrax apparently does not depend upon the persistence of primary chlorine, namely, chlorine in the form of either free chlorine, hypochlorous acid or hypochlorites. Effluents sufficiently chlorinated to become anthrax-free in less than two hours can receive much additional chlorine without showing evidence of "saturation," that is, without returning as "available chlorine" anywhere near 100 per cent of the added increment of chlorine. Nor do such mixtures bleach certain colors or indicator solutions nearly so rapidly as do very small concentrations of primary chlorine. For the most part, then, disinfection of the effluents is apparently effected by N-chloro compounds produced through the reaction of chlorine with nitrogenous substances originally contained therein.

The simplest N-chloro compound is monochloro-amine, derived from ammonium ions and hypochlorite ions, as in the equation,



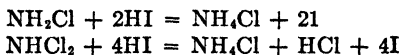
Methylamine ( $\text{CH}_3\text{NH}_2$ ) similarly yields monochloro-methylamine ( $\text{CH}_3\text{NHCl}$ ), while glycine ( $\text{NH}_2\text{CH}_2\text{CO}_2\text{H}$ ) affords monochloro-glycine ( $\text{NHCl}\cdot\text{CH}_2\text{CO}_2\text{H}$ ). But these three monochloro derivatives are permanent only in solutions of a certain degree of alkalinity. Under the influence of acid they become converted at varying rates into the dichloro derivatives. Thus, below pH

8.5, most rapidly and extensively near pH 4.5, monochloro-amine affords dichloro-amine according to the equation,



Similarly monochloro-methylamine affords dichloro-methylamine ( $\text{CH}_3\text{NCl}_2$ ) at pH 1.8 to 9.5, while monochloro-glycine yields dichloro-glycine ( $\text{NCl}_2 \cdot \text{CH}_2\text{CO}_2\text{H}$ ) at pH 1.9 to 7.8, the rate and extensiveness of the conversion increasing with increasing acidity, so that between the extremes very varying mixtures may occur. Dichloro-amine below pH 4.4 becomes further converted to nitrogen trichloride ( $\text{NCl}_3$ ).

When dilute solutions of these chloro derivatives are assayed by treatment with acidified potassium iodide and subsequent titration with sodium thiosulphate, the mono- and dichloro products, like hypochlorites, afford "available chlorine" in twice the proportion of the constituent chlorine, as in the equations,



Nitrogen trichloride should behave similarly but interference by a side reaction cuts the yield to about 80 per cent of the theoretical. For practical reasons the concentrations of all the substances are expressed in the following tables on the common basis of parts per million available chlorine actually recoverable by the iodide-thiosulphate test, but the above relations must be taken into account in a final analysis of the data. Fuller discussion of the chemistry of the products will be found in recent papers by one of the writers (Chapin, 1929).

Ammonia is known to be present in soak waters from hides and skins. Methylamine or its homologues has been identified therein by McLaughlin, Highberger and Moore (1928). Glycine was selected for use merely as a convenient type of the amino acid undoubtedly occurring, either separate or conjugated, in soak waters through the degradation of protein.

#### PREPARATION OF THE SUBSTANCES

All N-chloro-derivatives were prepared by rapid admixture of appropriately buffered solutions of chlorine with the nitrogenous

substance, the latter being in at least 100 per cent excess. The final pH, length of storage and other details were such as Chapin (1929) showed necessary to insure, in each case, the production of a single chloro derivative, not a mixture. All solutions were assayed for available chlorine as shortly as practicable before use in the bacteriological tests.

*Chlorine water* was prepared by dissolving the washed gas evolved from a mixture of potassium permanganate or manganese dioxide and diluted hydrochloric acid in an amber bottle of distilled water immersed in an ice bath. It contained roughly about 0.5 per cent chlorine, and was kept under refrigeration in the dark.

*Monochloro-amine* was prepared by mixing equal volumes of a solution containing 0.2 gram ammonium sulphate with 40 cc. M/10 borax per 100 cc. and of a solution containing 2 cc. N sodium hydroxide with not over 10 cc. chlorine water per 100 cc. The final reaction was slightly above pH 9.0.

*Dichloro-amine* was prepared by mixing equal volumes of a solution containing 0.75 gram ammonium acetate with 2.5 cc. N hydrochloric acid and of a solution containing 6.5 cc. M sodium acetate, 3.5 cc. M acetic acid and not over 25 cc. chlorine water per 100 cc. The mixture was stored at room temperature at least 2 hours before use. Its final reaction was near pH 4.8.

*Nitrogen trichloride* was prepared by passing chlorine gas into a solution of ammonium sulphate under chloroform (Noyes, 1920). A little of the chloroform solution, freshly washed with dilute ammonium sulphate, was vigorously shaken with at least 500 times its volume of 0.1 per cent ammonium sulphate to afford an aqueous solution for test, the chloroform completely passing into solution.

*Monochloro-methylamine* was prepared by mixing equal volumes of a solution containing 3 cc. M methylamine, 30 cc. M/10 borax and 4 cc. N sodium hydroxide per 100 cc. and of a solution containing the same quantities of borax and sodium hydroxide with 12 cc. chlorine water per 100 cc. The final pH was nearly 10.2.

*Dichloro-methylamine* was prepared by mixing equal volumes of a solution containing 2 cc. M methylamine, 25 cc. M/2 dihydrogen potassium phosphate and 14.4 cc. M/2 phosphoric acid

per 100 cc. and of a solution containing the same quantities of phosphate and phosphoric acid with 10 cc. chlorine water per 100 cc. The final mixture was stored at least three hours before use. Its reaction was about pH 2.4.

*Monochloro-glycine* was prepared by mixing equal volumes of a solution containing 0.2 gram glycine, 31.6 cc. M/10 borax and 1.85 cc. N hydrochloric acid per 100 cc. and of a solution containing the same quantities of borax and hydrochloric acid with 12 cc. chlorine water per 100 cc. The final reaction was nearly pH 8.4.

*Dichloro-glycine* was prepared with the same buffer mixture as dichloro-methylamine, but with 0.4 gram glycine and 30 cc. chlorine water in the respective foundation solutions. These were cooled before mixing and the product was stored for three hours in an ice bath. After being extracted twice with 10 cc. carbon tetrachloride to remove traces of nitrogen trichloride and blown with air to expel carbon tetrachloride it was brought to room temperature and employed as soon as possible. Its final reaction was near pH 2.2.

#### BACTERIOLOGICAL WORK

The technique employed in these tests was as follows: The surface growth of agar cultures of *B. anthracis* from two to three weeks old was suspended in sterile normal saline solution and the stock suspension thus prepared was kept in the refrigerator. For the purposes of the tests the stock suspension was diluted with sterile distilled water so as to contain about 1,000,000 spores per cubic centimeter and 1 cc. of this dilution was added to 9 cc. of disinfectant and the mixture held at room temperature. At appropriate intervals subcultures were made into tubes of beef infusion broth adjusted to neutrality to phenolphthalein and the subculture tubes were then incubated at 37.5°C. for forty-eight hours.

At the beginning of this work comparative tests were made using subculture tubes of the previously mentioned culture medium, with and without the addition of sodium thiosulphate. These tests indicated that the neutralizing capacity of the culture

TABLE 1  
*Bactericidal efficiency of chlorine in neutral, acid and alkaline solutions*

REACTION	AVAILABLE CHLORINE	GROWTH AFTER (TIME OF EXPOSURE)	NO GROWTH AFTER (TIME OF EXPOSURE)
	<i>p.p.m.</i>		
Neutral	2	45 minutes	
Neutral	3	30 minutes	45 minutes
Neutral	4	15 minutes	30 minutes
Acid	2	45 minutes	
Acid	3	15 minutes	30 minutes
Acid	4		15 minutes
Alkaline	100	2 hours	

Test organism, spores of *B. anthracis* Strain No. 6.

TABLE 2  
*Bactericidal efficiency of chlorine in buffer solutions*

pH	AVAILABLE CHLORINE	EXPOSURE TIME				
		15 minutes	30 minutes	45 minutes	1 hour	2 hours
	<i>p.p.m.</i>					
10.0	100	+	+	+	+	+
9.0	20	+	+	+	+	+
	30	+	+	+	+	—
	40	+	+	+	—	—
8.0	50	+	+	—	—	—
	10	+	—	—	—	—
	10	+	—	—	—	—
7.0	10	+	—	—	—	—
	10	+	—	—	—	—
6.0	3	+	+	+	+	+
	4	—	—	—	—	—
5.0	2	+	+	+	+	+
	3	+	—	—	—	—
4.0	1	+	+	+	+	+
	2	+	+	—	—	—
3.0	1	+	+	+	+	+
	2	+	+	—	—	—

Test organism, spores of *B. anthracis* Strain No. 6. + = growth. — = no growth.

medium itself was sufficient to prevent inhibition of growth by the small amount of disinfectant carried over into the medium and therefore no thiosulphate was used in the subsequent tests.

Previous experience having shown that strains of *B. anthracis*



may differ considerably in resistance to disinfectants a number of strains were tested for their resistance to chlorine and three of the most resistant strains were used in these tests.

Experiments were first conducted to determine the bactericidal efficiency of chlorine against anthrax spores in neutral, acid and alkaline solutions. For neutral test dilutions freshly assayed chlorine water was diluted with sterile distilled water. The acid

TABLE 3

*Bactericidal efficiency of monochloro-amine, dichloro-amine and nitrogen trichloride*

DISINFECTANT	AVAILABLE CHLORINE	EXPOSURE TIME					
		15 minutes	30 minutes	45 minutes	1 hour	1½ hours	2 hours
	<i>p.p.m.</i>						
Monochloro- amine, pH 9.0	20	+	+	+	+	-	-
	40	+	+	+	-	-	-
	60	+	+	-	-	-	-
	80	+	+	-	-	-	-
Dichloro-amine, pH 4.8	20	+	+	+	+	-	-
	40	+	+	-	-	-	-
	60	+	+	-	-	-	-
	80	+	-	-	-	-	-
Nitrogen tri- chloride	2	+	+	+	+	+	+
	4	-	-	-	-	-	-
Chlorine	4	+	+	+	+	+	+
	6	-	-	-	-	-	-

Test organism, spores of *B. anthracis* Strain No. 14. + = growth. - = no growth.

and alkaline test dilutions contained in addition one-twenty-fifth their volumes of N/2 HCl or NaOH. For simplicity the final concentrations were termed N/50, without regard to reactions with chlorine.

Experiments were next conducted to determine the bactericidal efficiency of chlorine against anthrax spores in borate, phosphate and phthalate buffer solutions. In these experiments the stock chlorine solution was diluted with the appropriate standard buffer

solutions so as to adjust the reaction of the test dilutions to the desired pH. The results obtained are shown in table 2.

The experimental technique used in tests with nitrogen trichloride, dichloro-amine and monochloro-amine was the same as that employed in the preceding experiments. Test dilutions of

TABLE 4  
*Bactericidal efficiency of chlorine derivatives of methylamine and glycine*

DISINFECTANT	AVAILABLE CHLORINE	EXPOSURE TIME					
		15 minutes	30 minutes	45 minutes	1 hour	1½ hours	2 hours
Monochloro-methylamine, pH 10.2	p.p.m.						
	200	+	+	+	+	+	+
Dichloromethyl-amine, pH 2.4	10	+	+	+	+	+	+
	20	+	+	+	—	—	—
	30	+	+	—	—	—	—
	40	+	+	—	—	—	—
	50	+	—	—	—	—	—
Monochloro-glycine, pH 8.4	240	+	+	+	+	+	+
Dichloro-glycine, pH 2.2	10	+	+	+	+	+	+
	20	+	+	+	+	—	—
	30	+	+	+	+	—	—
	40	+	+	+	+	—	—
	50	+	+	—	—	—	—
Chlorine	6	+	+	+	—	—	—
	8	+	+	—	—	—	—
	10	—	—	—	—	—	—

Test organism, spores of *B. anthracis* Strain No. 15. + = growth. — = no growth.

the disinfectant were prepared just before the test by diluting a standardized stock solution with sterile distilled water. Nitrogen trichloride was found to be very unstable in aqueous solution and the various test dilutions were therefore used as quickly as possible so as to minimize the inevitable deterioration. Dichloro-amine

and monochloro-amine were found to be relatively stable in aqueous solution but even with these the test dilutions were used without any unnecessary delay. The results obtained are shown in table 3. Since a different strain of anthrax spores was employed results are also shown for chlorine.

The results of similar tests with monochloro and dichloro-methylamine and with monochloro and dichloro-glycine are shown in table 4.

#### SUMMARY

Chlorine, in neutral, acid and alkaline solutions, and the N-chloro derivatives of ammonia, methylamine and glycine have been tested against spores of *B. anthracis*. Nitrogen trichloride, chlorine in neutral solution, and chlorine plus N/50 HCl were effective in 15 minutes with available Cl concentrations of 10 p.p.m. or less. Chlorine plus N/50 NaOH was not effective in two hours with 100 p.p.m. available Cl. Monochloro-amine and dichloro-amine were effective with 80 p.p.m. available Cl in forty-five minutes and thirty minutes, respectively. Dichloro-methylamine and dichloro-glycine were effective with 50 p.p.m. available Cl in thirty minutes and forty-five minutes, respectively. Monochloro-methylamine and monochloro-glycine were not effective in two hours with 200 and 240 p.p.m. available Cl, respectively.

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# ON CERTAIN FACTORS INFLUENCING THE SURVIVAL OF BACTERIA IN WATER AND IN SALINE SOLUTIONS

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This paper deals with the survival of bacteria in the apparent absence of nutrient material (unwashed bacteria in 0.85 per cent NaCl solution and in distilled water); in the actual absence of nutrient material (washed bacteria in the same suspending fluids); and in the presence of varying amounts of nutrient material (with addition of broth to the above suspending fluids). It also contrasts bacterial survival in distilled water with survival in 0.85 per cent NaCl solution and deals with the influence of bacterial concentration on survival in the above fluids at various temperatures.

## LITERATURE

*Prolonged survival in water.* Most of the observations on survival of bacteria in water have been made upon bacterial concentrations insufficient to allow survival longer than 2 or 3 months (Strauss and Dubarry, 1889; Frankland, 1895; Wheeler, 1906; Livingstone, 1921). A few instances of much more prolonged survival in water have been recorded (*S. cholerae*, 7 months, Ficker, 1898; *B. typhosus*, 490 days, Konrádi, 1904). I have been unable to find any record of prolonged survival of bacteria in NaCl solution.

*Factors influencing survival in water and in saline solutions.* Among the factors which have been found to influence the survival of bacteria in aqueous suspensions, the following may be noted: the character of the water and particularly its content of organic matter (Bolton, 1886; Wheeler), temperature (Frankland;

Wheeler), diffuse light (Wheeler), sterilization of the water (Frankland; Wheeler), the amount of inoculum (Ficker; Livingstone), the age of the culture used in making the suspension (Ficker), the addition of minute amounts of culture media (Bolton), and previous prolonged contact of the water with metal (Ficker). In addition, Cohen (1922) and Winslow and Falk (1923) have extensively investigated the influence of the pH of water and of saline solutions on the survival of bacteria. Winslow and Falk (1918 and 1923), Panisset, Verge and Carneiro (1925), and Shaughnessy and Criswell (1925), and Duthôit (1923) have compared the viability of bacteria in distilled water with that in saline solutions of varying concentration. Ficker and Cohen have made some observations on the influence of substances absorbed into the water from glass containers. Whipple and Mayer (1906) have shown that lack of oxygen unfavorably influences longevity of *B. typhosus* in water. The viability of bacteria in water in nature, or under conditions intended to simulate those in nature has been studied by Houston (1912 and 1913-1914), Jordan, Russell and Zeit (1904), Russell and Fuller (1906), and others. The comparative viability of washed and unwashed bacteria in aqueous suspensions has been investigated by Winslow and Brooke (1927). The organisms whose survival in saline solutions has been most frequently studied are *B. typhosus*, *B. coli*, and *S. cholerae* while the survival of others such as *Strep. haemolyticus* (Livingstone), and *Staph. aureus* (Bolton; Panisset, Verge and Carneiro, and others) has also been investigated to some extent.

#### MATERIAL AND METHODS

The organisms employed in the following studies were laboratory stock cultures, most of which had been grown for prolonged periods on artificial media. Acid-cleaned, sterile, ordinary glass test tubes were used for storing bacterial emulsions to be tested for survival, the tubes being plugged with cotton stoppers, but not sealed. A mark was made to indicate the height of the fluid in the tubes, and loss by evaporation was subsequently made up to the mark from time to time, by the addition of sterile distilled

water. Chemically pure sodium chloride was used in making NaCl solutions, and in some of the later experiments Merck's blue label quality was employed. Both the saline solutions and the distilled water were sterilized in pyrex flasks, in the autoclave, at 15 pounds pressure, for 15 minutes.

*Colony counts* were made by pipetting and suitably diluting 0.01 cc. of the bacterial emulsion, and sowing, on nutrient agar pour plates, a quantity likely to yield 100 to 200 colonies. In all cases bacterial emulsions to be tested were first thoroughly agitated. Sodium chloride solution 0.85 per cent was used as the diluting fluid.

*Washing* was performed by centrifuging bacterial emulsions, twice resuspending and centrifuging them in distilled water or in 0.85 per cent NaCl solution, under aseptic precautions. When supernatant fluid was to be used, it was always obtained after the first centrifuging.

*Purity of emulsions of bacteria* of the colon-typhoid group was controlled by obtaining typical reactions on Russell media, from time to time. Gram stains were also made at various intervals from bacterial emulsions used in the experiments.

#### PROLONGED SURVIVAL

In this experiment bacterial emulsions were made by washing the growth off twenty-four-hour (*B. tuberculosis*, forty-eight-hour) agar slant cultures, making 10 cc. quantities of emulsion containing about 2 to 3 billion colony producers per cubic centimeter. These emulsions were kept in the dark and were tested for viability, from time to time, by sowing one loopful of emulsion on an agar slant.

1. *Prolonged survival in 0.85 per cent NaCl solution.* (a) At 37°C.: *B. pyocyaneus*<sup>3</sup> remained viable 30½ months. *B. paratyphosus* A<sup>2</sup> and B,<sup>2</sup> *B. enteridis*,<sup>2</sup> *B. coli-communis*,<sup>2</sup> *B. dysenteriae* Shiga,<sup>2</sup> *B. tuberculosis*,<sup>2</sup> and *Strep. viridans*<sup>2</sup> (*ignavus*) remained viable for 13½ months. *B. typhosus*<sup>1</sup> (strain 1) remained viable

<sup>1</sup> Found to be non-viable a few months later.

<sup>2</sup> Not tested later.

<sup>3</sup> Still under observation.

for 8 months, while *B. typhosus*<sup>1</sup> (strain 2, Rawlings) remained viable for 5 months. *B. mucosus-capsulatus*<sup>1</sup> survived 3½ months. (After 30½ months *B. pyocyaneus* still showed pigment production. After 13½ months *Strep. viridans* showed methemoglobin production.) (b) At room temperature: *B. typhosus*<sup>3</sup> survived 32 months. *B. pyocyaneus*<sup>3</sup> and *B. coli*<sup>3</sup> survived 31¼ months, and *B. paratyphosus* B<sup>1</sup> survived 21¼ months. (c) At 0° to 8°C.: *B. typhosus*<sup>1</sup> remained viable 25½ months; *B. pyocyaneus*,<sup>1</sup> 25 months; and *B. tuberculosis*,<sup>1</sup> 16 months.

2. *Prolonged survival in distilled water.* (a) At 37°C.: *B. pyocyaneus*<sup>3</sup> survived 30¼ months; *Strep. viridans*<sup>1</sup> (*ignavus*), 21¼ months; *B. tuberculosis*,<sup>1</sup> 16 months; and *B. typhosus*<sup>1</sup> (Rawlings), 14 months. (b) At room temperature: *B. typhosus*<sup>3</sup> (Rawlings) remained viable 32 months; *B. pyocyaneus*,<sup>3</sup> *B. coli*,<sup>3</sup> *B. mucosus-capsulatus*,<sup>3</sup> and *B. tuberculosis*,<sup>3</sup> 31¼ months; and *B. paratyphosus* B<sup>1</sup>, 25 months. (c) At 0° to 8°C.: *B. pyocyaneus*<sup>1</sup> and *Strep. viridans*<sup>1</sup> (*ignavus*) survived 25 months; *B. typhosus*<sup>1</sup> (Rawlings), 22½ months; *B. tuberculosis*<sup>1</sup>, 21¼ months; and *B. coli*<sup>1</sup> and *B. paratyphosus* B<sup>1</sup>, 16 months.

In my experience, survival of the organisms mentioned above has been prolonged much more uniformly in distilled water and in 0.85 per cent NaCl solution, than on solid media. Cultures of the same strain made at different times on solid media but stored under the same conditions may either die within 2 months or survive more than 6 months.

EFFECT OF BACTERIAL CONCENTRATION ON SURVIVAL AT VARIOUS TEMPERATURES, (1) IN 0.85 PER CENT NaCl SOLUTION, (2) IN DISTILLED WATER

In this series of experiments eighteen- to twenty-hour agar slant cultures of *B. typhosus* were washed off and emulsified; some, in distilled water, and others, in 0.85 per cent NaCl solution. From emulsions made in this way, a set of 10 dilutions was prepared for each experiment, with a bacterial concentration in each dilution 50 per cent of that in the preceding one, the first dilution in each case containing approximately 3 to 4 billion colony producers per cubic centimeter (10 cc. quantities in 18

mm. test tubes). One of these sets of emulsions in 0.85 per cent NaCl solution was stored at 37°C., another at 15° to 20°C., and

TABLE 1

*Colony count per cubic centimeter of emulsion of B. typhosus (Rawlings) after time indicated in 0.85 per cent NaCl solution at 37°C.*

DILUTION NUMBER	1 HOUR	24 HOURS	42 HOURS	4 DAYS	9 DAYS	15 DAYS	22 DAYS	29 DAYS	43 DAYS	56 DAYS
1	3075M	2700M	2260M	500M	76M	6.4M	1.6M	1.8M	1.3M	670T
2	1540M			1400M	10M	2.4M	580T	420T	410T	430T
3	870M	837M	250M	30M		1M	142T	155T	244T	96T
4	440M			1M	750T	74T	100			
5	220M	56M	7.5M	300T	200	0				
6	110M				10—					
7	60M	4M	266T	100—	0					
8	30M				1					
9	15M	400T	5T—	5—	1					
10	8 9M			0	0					

TABLE 2

*Colony count per cubic centimeter of emulsion of B. typhosus (Rawlings) after time indicated in 0.85 per cent NaCl solution at room temperature (15° to 20°C.)*

DILUTION NUMBER	5 HOURS	36 HOURS	8 DAYS	11 DAYS	15 DAYS	25 DAYS	36 DAYS	49 DAYS	88 DAYS	128 DAYS
1	4530M	2950M		2489M	1670M	410M	620M	35M	67M	55M
2	2260M									
3	1130M	1410M								
4	566M			405M	386M	185M	7M			1M
5	283M	277M								
6	141M			40M	48M	4M	180T	277T	240T	150T
7	70M	23M			6.4M		14T	18T	0	
8	35M				380T		10—			
9	17M				6T	100—				
10	8.8M		800	320	30	0				

M = millions; T = thousands; — = less than.

N.B.: Figures given in 1 hour and 5 hour columns are based on actual colony counts of dilutions 1 and 10.

a third at 0° to 8°C. Likewise one set of these emulsions in distilled water was stored at each of the above temperatures. Two sets (two different strains) of *B. typhosus* were placed in 0.85



per cent NaCl solution at 37°C. Colony counts were made at intervals by the method indicated above under "methods." The results are given in tables 1 to 6.

1. *Survival of B. typhosus in 0.85 per cent NaCl solution* (tables 1 to 4). Only 4 dilutions survived 15 days in 0.85 per cent NaCl solution at 37°C. (i.e., only those containing 440 million colony producers or more per cubic centimeter).<sup>4</sup> At room temperature all 10 dilutions survived for this period (the tenth containing 8.8 million colony producers per cubic centimeter); and at 0° to 8°C. 6 dilutions survived the 15 days

TABLE 3

*Colony count per cubic centimeter of emulsion of B. typhosus (Rawlings) after time indicated in 0.85 per cent NaCl solution in cold room (0° to 8°C.)*

DILUTION NUMBER	5 HOURS	36 HOURS	8 DAYS	11 DAYS	15 DAYS	25 DAYS	36 DAYS	49 DAYS	88 DAYS	128 DAYS
1	2944M	3650M	3220M	940M	3000M	2880M	1260M	1770M	129M	54M
2	1472M									
3	736M	860M								
4	368M			220M	312M	385M	50M			1.2M
5	184M	167M								400T
6	92M			40M	6 2M	2. 2M	180T	547T	120	
7	46M	20M		100—						
8	23M			10—						
9	11M	612T		0						
10	5.7M		0	0						

M = millions; T = thousands; — = less than.

N.B.: Figures given in 1 hour and 5 hour columns are based on actual colony counts of dilutions 1 and 10.

(i.e., those containing 92 million colony producers or more per cubic centimeter). Room temperature appears to be more favorable for the survival of *B. typhosus* in 0.85 per cent NaCl solution than 0° to 8°C., and much more favorable than 37°C.

<sup>4</sup> Evidence of the great resistance of *B. typhosus* in high bacterial concentration to relatively strong NaCl solutions was found in an experiment, in which *B. typhosus* (Rawlings) in concentrations of about 3 billion colony producers per cubic centimeter survived 2 weeks in both 8 and 4 per cent NaCl solution when kept in 10 cc. quantities at 37°C., and *B. typhosus* (strain 1) survived 2 weeks in 8 per cent and 2 months in 4 per cent NaCl solution.

An initial sowing of more than 220 million colony producers per cubic centimeter was required for survival beyond 9 days at 37°C.,

TABLE 4

*Colony count per cubic centimeter of emulsion of B. typhosus (strain I) after time indicated in 0.85 per cent NaCl solution at 37°C.*

DILUTION NUMBER	1 HOUR	24 HOURS	42 HOURS	4 DAYS	9 DAYS	15 DAYS	22 DAYS	29 DAYS	43 DAYS	56 DAYS
1	3025M	1450M	2120M	160M	20M	6.7M	2.6M	1.1M	778T	415T
2	1500M			210M	8M	4.2M	2M	650T	124T	182T
3	750M	450M	310M	20M		354 T	756T	70T	103T	480
4	375M			5M	470T	55	0			
5	188M	33M	14M	1.1M	14T	0				
6	94M				10—					
7	47M	1.6M	66T	100—	1					
8	23M				1					
9	11M	200T—	5T—	5—	0					
10	5.1M			2	0					

TABLE 5

*Colony count per cubic centimeter of emulsion of B. typhosus (Rawlings) after time indicated in distilled water at 37°C.*

DILUTION NUMBER	5 HOURS	36 HOURS	8 DAYS	11 DAYS	15 DAYS	25 DAYS	29 DAYS	38 DAYS	49 DAYS	128 DAYS
1	3072M	3100M	100M	14M	4.3M	13M		8.2M	8.7M	185T
2	1536M									4T
3	768M	750M						360T		10—
4	384M		23M	1M	1.9M	195T		233T	78T	10—
5	192M	182M					6T	10—		
6	96M			310T	62T	750	5—			
7	48M	31M					0			
8	24M					100—	0			
9	12M			2.6T	110	0				
10	6M		500	100	4					

M = millions; T = thousands; — = less than.

N.B.: Figures given in 1 hour and 5 hour columns are based on actual colony counts of dilution 1 and 10.

and more than 46 million for survival beyond 11 days at 0° to 8°C., while only 8 million were required for 15 days survival at 15° to 20°C.

An early period of high death rate of *B. typhosus* in 0.85 per cent NaCl solution was observed, as compared with a later period of comparatively low death rate. For example, in dilution 1 at 37°C. (table 1) at the end of the first 3 weeks only 1.6 million colony producers per cubic centimeter survived out of an original 3 billion (approximately 0.05 per cent). However, of this 1.6 million colony producers per cubic centimeter which survived 3 weeks, 1.3 million per cubic centimeter or approximately 80 per cent survived another 3 weeks. Also, in dilution 1 at room temperature (table 2) at the end of the first 88 days only 67 million

TABLE 6

*Colony count per cubic centimeter of emulsion of B. typhosus (Rawlings) after time indicated in distilled water at room temperature (15° to 20°C.)*

DILUTION NUMBER	5 HOURS	8 DAYS	11 DAYS	21 DAYS	28 DAYS	42 DAYS	60 DAYS	78 DAYS	100 DAYS
1	2682M	2200M			900M	205M	65M		62M
2	1350M								
3	680M								
4	345M	220M							3.6M
5	173M								
6	86M	90M							210T
7	43M								
8	21M								800
9	10M								7
10	5.4M		2.7M	2.1M	570T	33T	740	50	10—

M = millions; T = thousands; — = less than.

N.B.: Figures given in 1 hour and 5 hour columns are based on actual colony counts of dilutions 1 and 10.

colony producers per cubic centimeter survived out of an original 4.5 billion (approximately 1.5 per cent). On the other hand, of this 67 million colony producers per cubic centimeter remaining after 88 days, 55 million or approximately 82 per cent survived another 40 days. Similarly, in the case of dilution 1 at 0° to 8°C. (table 3) at the end of the first 88 days 129 million colony producers per cubic centimeter survived out of an original 3 billion (approximately 4.5 per cent), but, of this 129 million colony producers per cubic centimeter remaining after 88 days, 54 million or approximately 41 per cent survived for another 40 days.

Comparison of table 4 with table 1 shows that the results with the two strains of *B. typhosus* in 0.85 per cent NaCl solution at 37°C. were approximately the same.<sup>5</sup>

2. *Survival of B. typhosus in distilled water* (tables 5 and 6). Survival of *B. typhosus* (Rawlings) in distilled water at 37°C. (table 5) was very much more prolonged in the weaker bacterial concentrations, than in bacterial concentrations of approximately equal strength in 0.85 per cent NaCl solution at the same temperature (table 1). All 10 dilutions remained viable in distilled water for 15 days, while only 4 survived for this period in 0.85 per cent NaCl solution, although each dilution in 0.85 per cent NaCl solution was of slightly higher bacterial concentration than the correspondingly numbered dilution in distilled water. Dilution 1 in distilled water remained viable for 14 months, while dilution 1 in 0.85 per cent NaCl solution remained viable for only 5 months.

Survival was still more prolonged in distilled water at room temperature (table 6) than in distilled water at 37°C. Dilution 10 at room temperature yielding 740 colony producers per cubic centimeter after 60 days, out of about 6 million per cubic centimeter at the commencement of the experiment, while at 37°C. all dilutions containing less than 190 million colony producers per cubic centimeter at the commencement of the experiment were non-viable after 36 days. Survival in distilled water at room temperature was also much more prolonged than in 0.85 per cent NaCl solution at room temperature (cf. table 6 with table 2).

The table showing the results with *B. typhosus* in distilled water at 0° to 8°C. has been omitted to save space; however, 2 million colony producers per cubic centimeter remained in dilution 10 after 30 days, out of an original 6 million colony producers per cubic centimeter at the commencement of the experiment. This dilution was still viable after 94 days. This is a much more pro-

<sup>5</sup> A similar experiment was conducted with *B. coli* and *B. paratyphosus* B. These organisms showed ability to survive in much lower bacterial concentration than *B. typhosus* under similar circumstances. With an initial colony count of 52 million per cubic centimeter *B. paratyphosus* B survived 28 days and *B. coli-communis* survived 21 days in 0.85 per cent NaCl solution at 37°C.

longed survival than was obtained in 0.85 per cent NaCl solution at 0° to 8°C. (see table 3). Still weaker concentrations of *B. typhosus* in distilled water were tested later at 0° to 8°C., one set made with washed, the other with unwashed bacteria. Concentrations of about 2 million colony producers per cubic centimeter (whether washed or unwashed) remained viable for 50 days, those of about 2000 to 200,000 (washed or unwashed) remained viable for about 3 weeks, and those of 50 to 1200 remained viable for 4 to 13 days. No effect of washing was apparent in this experiment. (See discussion.)

An early period of high death rate succeeded by a later period of comparatively low death rate was also observed in the case of *B. typhosus* in distilled water. Dilution 1 in distilled water at room temperature (table 6) furnishes an example of this phenomenon. In this case the number of colony producers surviving after 42 days was approximately 8 per cent of the number present at the commencement of the experiment, while, of the 205 million which survived for 42 days, 62 million or approximately 30 per cent survived for another 58 days.

#### SURVIVAL OF WASHED BACTERIA, EFFECT OF MATERIAL REMOVED IN WASHING

In these experiments emulsions of *B. typhosus* were made by washing off eighteen- to twenty-hour agar slant cultures. These emulsions were centrifuged, the supernatant fluid was removed, and saved where required. The bacterial sediment was resuspended and recentrifuged, and the supernatant fluid discarded. Finally, the sediment was resuspended to make an emulsion of washed bacteria. Either distilled water or 0.85 per cent NaCl solution was used exclusively throughout each experiment.

Table 7 shows that the washed bacteria (both strain 1 and strain 2) were all dead in 42 days, while both the unwashed controls still showed more than 50 thousand colony producers per cubic centimeter after 80 days. The experiment with emulsion WSF, table 8 shows that when the material washed from the bacterial emulsions is restored to them, they will survive as well as the unwashed controls.

TABLE 7

*Wash experiment 1. Colony count per cubic centimeter of washed and of unwashed emulsion of B. typhosus (strain 1) and (strain 2, Rawlings) after time indicated in 0.85 per cent NaCl solution at 37°C.*

	5 HOURS	7 DAYS	13 DAYS	35 DAYS	38 DAYS	42 DAYS	80 DAYS
Strain 1 {	U	2500M	46M	28M	1.8M	830T	57T
	W	2200M	16M	9M	2T—	10	0
Strain 2 {	U	1700M	106M	47M	14M	9M	827T
	W	2800M	8M	2.7M	1T	10—	

TABLE 8

*Wash experiment 2. Colony count per cubic centimeter of emulsion of B. typhosus (strain 2, Rawlings) in 0.85 per cent NaCl solution at 37°C. after time and preparation indicated*

	5 HOURS	2 DAYS	15 DAYS	18 DAYS	22 DAYS	42 DAYS	49 DAYS	56 DAYS	
Strain 2 {	U	4000M	1500M	11M	10—	7M	2.7M	510T	2.5M
	WS	3300M	100M	10T—		0			
	WSF	6400M	350M	1.7M		5M	3.8M	5.2M	2.6M
	SF	40M	20M	1.9M		1M	800T	2M	1.6M

TABLE 9

*Wash experiment 3. Colony count per cubic centimeter of washed and of unwashed emulsions of B. typhosus (Rawlings) after time indicated in distilled water at 37°C.*

DILUTION NUMBER	3 HOURS	6 DAYS	13 DAYS	16 DAYS	26 DAYS	33 DAYS	42 DAYS	85 DAYS	119 DAYS
U {	1	2600M		36M	2.9M	1.6M	1.5M	265T	70T
	2	236M		1.6M	56T	45	0		
	3	20M	2T	109					
	4	1.9M	2T	0					
W {	1	2850M		650T	700	36	0		
	2	258M		2.7T	0				
	3	22M	260	1					
	4	2.1M	6T	47	0				

U = unwashed; W = washed; WS = washed bacterial sediment resuspended in 0.85 per cent NaCl solution; WSF = washed bacterial sediment resuspended in supernatant fluid removed after first centrifuging; SF = supernatant fluid removed after first centrifuging; M = millions; T = thousands; — = less than.

Supernatant fluid removed during washing of an emulsion is really a very dilute emulsion of bacteria in the undiluted soluble constituents of the original emulsion. It is not surprising, therefore, that the bacteria present in supernatant fluid in comparatively small numbers should survive in such a favorable medium for a period out of all proportion to their numbers. This was what actually happened (emulsion SF, table 8). Similar results were obtained when another similar supernatant fluid was set up in a series of 10 dilutions. The protocol of the latter has been omitted to save space, but a summary of the result follows. With an initial 82 million colony producers per cubic centimeter in dilution 1 and 41 million in dilution 2, dilutions 1 and 2 both showed a colony count of over 40 thousand after 14 days, and 10 colonies each after 29 days. This is more than 3 times as long as the period for which dilutions of similar bacterial concentration survived when the soluble constituents of the emulsion were diluted to the same extent as the bacteria (dilutions 6 and 7 of tables 1 and 4).

The effect of washing is equally manifest when distilled water is used as the washing and suspending fluid instead of 0.85 per cent NaCl solution (cf. table 9 with tables 7 and 8). Practically no difference in duration of survival of washed and unwashed bacterial emulsions in distilled water was noted when the bacterial concentration was not greater than 22 million colony producers per cubic centimeter, but a decided difference was noted when the bacterial concentration reached 236 million. This corresponds with the results of a previous experiment (see above) in which no effect of washing was obtained using low bacterial concentrations in distilled water at 0° to 8°C. (See discussion.)

From these washing experiments, two facts are clearly demonstrated: first, the removal from a bacterial emulsion, of its soluble constituents results in relatively early death of the resuspended bacteria; and, second, a much reduced bacterial population, in the undiluted soluble constituents of an emulsion results in relatively long survival. However, the presence of the soluble constituents of an emulsion in an undiluted or relatively undiluted state is insufficient altogether to compensate for a great reduction in bacterial population.

ADDITION OF BROTH TO EMULSIONS OF *B. TYPHOSUS*, EFFECT ON SURVIVAL

In one of these experiments varying amounts of broth from 0.0005 to 1 cc. were added to 10 cc. emulsions of *B. typhosus* in 0.85 per cent NaCl solution containing about 300 million colony producers per cubic centimeter, and the emulsions were then placed at 37°C. Another similar experiment was conducted using larger proportions of broth from 10 to 100 per cent.

It was found that plain nutrient beef heart infusion broth in the proportion of one in a thousand was sufficient definitely to prolong the survival of *B. typhosus* in emulsion in 0.85 per cent NaCl solution at 37°C., and that 10 to 20 per cent plain nutrient broth was the optimum concentration for prolonged survival. Emulsions containing more or less than this proportion died out much more quickly.

## SURVIVAL OF VARIOUS ORGANISMS IN DISTILLED WATER, AND IN NaCl SOLUTIONS

In these experiments emulsions of various organisms containing about 300 million colony producers per cubic centimeter in distilled water, and in 0.4, 0.85 and 1.5 per cent NaCl solution were tested for survival at room temperature, at 0° to 8° and at 37°C.

The results indicated that most organisms tested will live as long or longer in distilled water and in 0.4 per cent NaCl solution than in 0.85 per cent NaCl solution, and usually longest in distilled water; such organisms included: *E. paratyphosus* B, *B. coli*, *B. mucosus-capsulatus*, *B. tuberculosis*, *B. diphtheriae*, *B. pertussis*, 2 strains of *Strep. hemolyticus* (*pyogenes*, one freshly isolated), *Strep. viridans* (*ignavus*), *Strep. viridans* (*salivarius*, freshly isolated), and 2 strains of *Staph. aureus* (one freshly isolated). Survival was uniformly shorter in 1.5 per cent than in 0.85 per cent NaCl solution at all 3 temperatures for all organisms tested. The only solution in which *B. pyocyaneus* failed to survive for 25 months at all 3 temperatures was 1.5 per cent NaCl solution. Most organisms survived longer at room temperature than at 0° to 8°C., although the streptococci, *B. diphtheriae* and a recently isolated strain of *B. mucosus-capsulatus* were exceptions to this rule.



### IS 0.85 PER CENT NaCl TOXIC TO ORGANISMS STORED IN NUTRIENT MEDIA?

Various organisms were grown in plain nutrient heart infusion broth containing respectively: no NaCl, 0.4 per cent NaCl and 0.85 per cent NaCl. These cultures were stored at 37°C. *B. typhosus* survived more than 3 times and *B. coli* more than twice as long in the broth containing no salt as in the broth containing 0.85 per cent NaCl. Survival in broth containing 0.4 per cent NaCl was of intermediate duration. *B. pertussis* and *B. tuberculosis* also survived definitely longer in the salt free broth than in that containing 0.85 per cent NaCl. Other organisms tested showed irregularly variable results.

### DISCUSSION

*Effect of glassware on reaction.* Probably the fact that pyrex glassware was used entirely for sterilizing distilled water and saline solutions in the above experiments has been of some importance, since Esty and Cathcart (1921) have shown that when such liquids are sterilized by steam in ordinary soft glassware they become alkaline, but when heated in pyrex glassware they become acid; and slight acidity favors survival according to Winslow and Falk (1923).

*Buffer effect of bacteria.* Probably of even greater importance has been the buffering action exerted by bacteria in water and in NaCl solutions, which tends to keep the reaction in a zone favorable for survival, as shown by Shaughnessy and Falk (1924) and Shaughnessy and Winslow (1927). The former authors found that this buffering action was greater in distilled water than in NaCl solutions. This corresponds with my finding that survival of bacteria is more prolonged in distilled water than in NaCl solutions, and offers a probable explanation of that result.

*Protective substances and effect of washing.* The so-called water of condensation on agar slant media is undoubtedly an important factor in survival of unwashed bacteria in emulsions either in water or in NaCl solutions, since it is carried over in all cases where emulsions are made by washing the growth off agar slants, and since Healy (1926) has shown that it is richer in albumen and

total solids than nutrient broth. The water of condensation may indeed be the most important factor removed by washing. In this connection it is interesting to note that Winslow and Brooke have demonstrated the protective influence of broth as well as peptone or meat extract upon washed emulsions of *B. cereus*. As they contend the effect is probably not a nutritive one. The results of Winslow and Brooke differ from those here reported in that they found the survival of *B. coli* to be uninfluenced by washing. This is readily explained by the fact that their experiment was of short duration and bacterial concentrations used were comparatively low, whereas I found the result of washing manifesting itself with *B. typhosus* after days rather than hours and not manifesting itself at all in low bacterial concentrations. (One would expect *B. coli* to be influenced by washing in a manner somewhat similar to *B. typhosus*.) Great dilution of the soluble products of an emulsion of bacteria may be almost equivalent to their total removal. This probably explains why no effect of washing was noted at low bacterial concentration (see experiment conducted at 0° to 8°C. and also see dilutions 3 and 4, table 9).

*Factors favoring survival of bacteria.* Low temperature, a minimum of nutrient material, a minimum or absence of sodium chloride, and a very high bacterial concentration are factors which favor survival of bacteria, but are unfavorable for bacterial growth. It is therefore necessary to differentiate clearly between those conditions which are favorable for growth and those which are favorable for survival.

The marked protective influence of low temperature (room temperature or lower) upon *B. typhosus* in low concentration in distilled water as demonstrated by the results of this investigation indicates that low temperature is probably of very great importance in the production of the winter river typhoid in temperate climates referred to by Hill (1911), although probably, interference with sunlight and diffuse light (Wheeler) by snow covered ice is also a factor. Similarly, my results add further support to the conclusion of Hinds (1917), that low temperature is to be considered an important factor in the results of Ruediger (1911) on summer and winter differences in polluted river water.

My finding that distilled water is a more favorable medium for the survival of *B. typhosus* and *B. coli* than 0.85 per cent NaCl solution corresponds with the results of Panisset, Verge and Carneiro and of Shaughnessy and Criswell with *B. coli*, but not with those of Winslow and Falk (1923) with *B. coli*, nor with those of Duthóit with *B. coli* and *B. typhosus*. It is worthy of note that the experiments of the authors quoted were all of short duration (only a few days at most), while mine were of long duration (from several days to many months). Consequently different factors might come into operation.

#### SUMMARY AND CONCLUSIONS

1. Various bacterial emulsions have remained viable for periods ranging from 5 to 32 months in 0.85 per cent NaCl solution at various temperatures, and from 14 to 32 months in distilled water.

2. The bacterial concentration required for survival of *B. typhosus* in emulsion in distilled water or in 0.85 per cent NaCl solution for a given time at a given temperature can be approximately determined. This varies greatly with the temperature and emulsifying fluid employed.

3. Distilled water is a more favorable medium for the survival of *B. typhosus* than 0.85 per cent NaCl solution. The same thing is true for a number of other organisms, including: *B. coli*, *B. tuberculosis*, *B. diphtheriae*, *Strep. hemolyticus* and *Strep. viridans*.

4. The prolonged survival of *B. typhosus* in distilled water and in 0.85 per cent NaCl solution is associated with a late period of comparatively low death rate. In the case of *B. typhosus* at 37°C. the onset of a period of low death rate may be detected after about 3 weeks.

5. The fact that *B. typhosus* in distilled water is able to survive so much longer (5 times as long in my experiment) at room temperature than at 37°C. points to low temperature as a factor of considerable importance in the production of winter river typhoid in temperate climates.

6. Washing *B. typhosus*, either in 0.85 per cent NaCl solution,

or in distilled water shortens its survival period when resuspended in these fluids. The supernatant fluid removed in washing prolongs the survival period of the relatively few bacteria which are inevitably present in it. Great dilution of an emulsion of bacteria appears to be almost equivalent to washing in its effect.

7. Ten to 20 per cent plain nutrient broth diluted with 0.85 per cent NaCl solution is the optimum concentration of broth for prolonged survival of *B. typhosus* at 37°C.; 0.1 per cent is sufficient definitely to prolong survival.

8. Sodium chloride in 0.85 per cent concentration appears to be toxic to certain organisms when stored in nutrient media at 37°C. Reduction in the concentration of sodium chloride in culture media might assist in the preservation of cultures of certain delicate organisms.

9. Room temperature appears to be more favorable for the survival of most organisms in distilled water and in 0.85 per cent NaCl solution than 0° to 8°C. The streptococci and *B. diphtheriae* are exceptions.

10. The duration of survival of several of the Gram negative bacilli appears to be more uniformly long in distilled water and in NaCl solutions than it is on solid media.

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# AN ORGANISM FOUND IN THE LATEX OF HEVEA BRASILIENSIS

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When an incision is made in the bark of the rubber tree (*Hevea brasiliensis*, Muell. Arg.), a somewhat viscous, creamy-white liquid, called latex, exudes. In the course of a few hours the liquid thickens and, later, a clot of rubber is formed which floats on the surface of a clear or milky serum. It is found that while the serum is acid, the surface layer is alkaline in reaction.

Much discussion has taken place concerning the biochemical changes occurring during the natural coagulation of *Hevea* latex. Whitby (1912) considered them to be due to enzyme activity while Eaton and Grantham (1915) advanced an explanation attributing them to the presence of bacteria. Both these views have received support from later workers and, more recently, de Vries and Beumée-Nieuwland (1924) have concluded that both bacteria and enzymes are responsible for the changes. It was recognised by Eaton and Grantham that precipitation of the rubber particles occurs as a result of the acid produced by bacterial activity but de Vries and Beumée-Nieuwland have shown that this precipitation is of the nature of flocculation and they consider that the subsequent coalescence of the particles (coagulation) is effected by a specific enzyme. Their views regarding the presence of an enzyme, however, have not met with general acceptance and Groenewege (1924), Belgrave (1925) and van Harpen (1929) offer a different explanation.

A bacteriological study of *Hevea* latex was carried out by Dernier and Vernet (1917) in Annam. These authors isolated 27 different species of bacteria of which one, "Bacillus No. 1,"

## CHARACTERISATION OF ORGANISM

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BRIEF CHARACTERISATION	REMARKS
<p>Microscopic features; Form: 5 (rods) Endospores: 2 (excentric to terminal)</p> <p>Flagella: 1 (peritrichic)</p> <p>Gram stain: 1 (positive)</p> <p>Vegetative cells: Diameter: 2 (between 0.5 and 1<math>\mu</math>) Length: 2 (more than 2 diameters)</p> <p>Chains: 1 (present)</p> <p>Capsules: 1 (present)</p> <p>Spores: Shape: 2 (ellipsoid to cylindrical) Diameter: 2 (greater than diameter of rod)</p> <p>Miscellaneous biochemical reactions: Biologic relationship: U (undetermined) Relation to oxygen: 2 (facultative anaerobe) Gelatin liquefaction: 1 (positive) In nitrate media: 2 (nitrite but no gas)</p>	<p>Some trouble was experienced in detecting endospores. The organism was not killed by maintaining cultures at 85°C. for 10 minutes. The spores were eventually found by Anjeszky's method using boiling carbol-fuchsin.</p> <p>The organism was found to be motile in agar culture. Flagella were observed by means of Loeffler's flagella stain.</p> <p>Organisms from cultures 3 to 5 days old were found to be Gram-positive by staining with gentian violet and counterstaining with carbol-fuchsin.</p> <p>Measurements were made from agar cultures stained with carbol-fuchsin. The rods, which had slightly rounded ends, had an average length of 3.1<math>\mu</math> and an average diameter of 0.9<math>\mu</math>. The largest cell measured had a length of 4.6<math>\mu</math> and a diameter of 1.2<math>\mu</math>.</p> <p>Chains were not common but occasionally chains containing up to 9 cells were seen.</p> <p>Capsules were detected by Hiss' method.</p> <p>The ellipsoid spores had an average length of 2.3<math>\mu</math> and an average diameter of 1.4<math>\mu</math>.</p> <p>Growth under anaerobic conditions similar to under aerobic conditions.</p> <p>Tests for nitrite were made by means of sulphanilic acid and <math>\alpha</math>-naphthylamine in acetic acid. The nitrate used gave no reaction with this test.</p>

<p>Chromogenesis: 0</p> <p>Carbohydrate reactions:</p> <p>Diastatic action: 0</p> <p>From d-glucose: 2 (acid without gas)</p> <p>From lactose: 1 (acid and gas)</p> <p>From sucrose: 1 (acid and gas)</p> <p>Cultural features:</p> <p>Agar stroke</p> <p>Growth: 2 (moderate)</p> <p>Lustre: 2 (dull)</p> <p>Surface: 1 (smooth)</p> <p>Agar colonies: 1 (punctiform)</p> <p>Gelatin colonies: 1 (punctiform)</p> <p>Relation of growth to reaction of medium:</p>	<p>A very pale straw colour was obtained with growth on agar.</p> <p>Litmus was completely decolourised in 2 to 3 days.</p> <p>The agar stroke was opaque, without chromogenesis or odour and no visible changes occurred in the medium.</p> <p>Agar colonies smooth and white, edge irregular. Growth slow.</p> <p>Gelatin colonies white, edge smoother than in the case of agar colonies. Growth slow.</p> <p>The organism grew in a dilute aqueous solution of egg albumen containing small amounts of mineral salts between pH = 4.0 and pH = 10.0. Good growth, however, only occurred between pH = 4.75 and pH = 9.0. It was difficult to delimit the pH range accurately as growth became very irregular as the end-points were approached.</p> <p>Complete coagulation of milk occurred 2 days after inoculation.</p>
<p>Milk:</p> <p>Acid: 1 (sufficient for curdling)</p> <p>Rennet curd: 1 (present)</p> <p>Peptonisation: 0 (absent)</p> <p>Indol production: 1 (positive)</p> <p>Additional reactions:</p> <p>Ammonia production: (positive)</p> <p>Hydrogen sulphide production: (negative)</p> <p>Albumen digestion: (positive)</p>	<p>When heated with magnesium oxide, the vapour from a 13 day old culture containing peptone and potassium nitrate turned litmus blue.</p> <p>Only a very slight darkening of lead acetate agar occurred after 12 days.</p> <p>Egg albumen was partially digested by cultures of the organism, the solution became alkaline and ammonia was evolved.</p>



CHARACTERISATION OF ORGANISM—*Concluded*

BRIEF CHARACTERIZATION	REMARKS
<p>Reactions with <i>Hevea</i> latex products:  <b>Latex:</b> (floculated)</p>	<p>Solutions were prepared by adding 1 part of latex to 9 parts of boiling water and heating the mixture at 15 pounds pressure for 15 minutes. After inoculation with the organism, the latex floculated, the surface layer being alkaline while the serum was acid. In most cases the further stage of coalescence was reached after 2 or 3 weeks. Similar results were obtained with more concentrated solutions of latex but coagulation occurred more readily.</p>
<p><b>Latex serum:</b> (acid without gas)  <b>Latex "carbohydrate:"</b> (acid without gas)</p>	<p>The latex "carbohydrate," presumably methyl-l-inositol (see Pickles and Whitfield, 1911), was prepared by evaporating latex serum to small bulk, taking up with alcohol, and evaporating the alcoholic solution to dryness. After inoculation of aqueous solutions of this substance with the organism, acid was produced without gas evolution.</p>

All work was carried out at the laboratory temperature which ranged between 26° and 30°C.

was present in all samples examined. "Bacillus No. 1" was described as an aerobic, motile, Gram-negative organism, producing acid with "dambosite" (the latex "carbohydrate"), lactose, and sucrose, effecting coagulation of milk and liquefaction of gelatin but unable to digest albumen.

The writer has isolated a bacillus from Hevea latex and a detailed study of its characteristics has been made. It was found in large numbers in all samples of latex examined from Selangor, Perak and Johore so that it appears to be distributed throughout the Malay Peninsula. It was invariably the predominant feature in samples which were coagulating and there can be no doubt that it is responsible for the important change occurring during the natural coagulation of Hevea latex.

The characteristics and reactions of the organism are given in detail in the manner required by the Committee on Bacteriologic Technic of the Society of American Bacteriologists (1924).

From the reactions given it will be seen that the organism in question can effect the precipitation of rubber from Hevea latex. The actual mechanism of the change is discussed in another paper (Corbet, 1929).

The organism differs from the "Bacillus No. 1" of Dernier and Vernet only in being Gram-positive and in its ability to digest albumen, so that the two may be identical.

The organism is a member of the genus *Bacillus* of the family Bacillaceae (see Bergey, 1926). It resembles closely several species of *Bacillus* which occur in the soil but does not appear to be identical with any one of them. It is most nearly allied to *Bacillus tumescens* (Zopf) and *B. silvaticus* (Neide) but differs from them in several important respects. The rapid decolorisation of litmus, which is so noticeable with the latex organism, would surely have been recorded had it been observed and no mention of this point is made in connection with *B. tumescens* or *B. silvaticus*.

As the organism does not appear to have been described previously, I propose to denominate it *Bacillus pandora*.

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## STUDIES ON RESPIRATORY DISEASES

### XXXIV. SOME RELATIONS BETWEEN EXTRACTS, FILTRATES AND VIRULENCE OF PNEUMOCOCCI<sup>1</sup>

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#### INTRODUCTION

The pathogenesis of pneumococcus pneumonia has been studied by a large number of workers, but is still only slightly understood. An approach to the study of the subject has been made by observing the influence of various products of the pneumococcus on the virulence of the pneumococcus *in vitro* and *in vivo*; by observing the pathological lesions produced by the pneumococcus and some of its products; and by noting the influence of several chemical reagents on the virulence of the pneumococcus. A pathological study of the tissues is reported in a paper (Pittman and Southwick) immediately following this one.

Because of the enormous number of contributions that have been made to the subject of pneumococcus infections, only a selected number can be mentioned here. Reference is given to those which include especially pertinent observations or bibliographic reviews.

It is now generally accepted that the "virulent" pneumococcus usually produces a smooth colony, has a capsule, produces type-specific soluble substances, is not easily agglutinated, has a high electrophoretic potential and is not readily engulfed by phagocytes; while "avirulent" organisms usually produce a rough colony, do not have a capsule, are easily agglutinated, have a

<sup>1</sup> The two articles on respiratory diseases in this issue are part of a series of studies carried out in connection with the Influenza Commission established and financially aided by the Metropolitan Life Insurance Company of New York.

low electrophoretic potential, and are readily phagocytized. In a discussion of microbic virulence one of us has cited the literature relating to these points (Falk (1928)).

The conversion of avirulent to virulent organisms and *vice versa* has been reported in a number of papers. The former process of conversion of the pneumococcus *in vitro* has not been as successful as with some other bacteria [e.g., paratyphoid cultures (Jordan, 1926) and *Bact. phaseoli-sojense*, (Rane (1929))]. Wadsworth and Kirkbride (1918) and Felton and Dougherty (1924) were unable to increase significantly the virulence by rapidly transferring the pneumococcus in broth or in skimmed milk.

Dawson and Avery (1927) and Dawson (1928) changed the R to the S forms by the use of anti-R serum but the most successful conversions have been accomplished by *in vivo* methods. The method that gave the best results, preceding the publication of Griffith's (1928) method was that of rapidly transferring the R organism through mice (Falk and Jacobson (1927), Dawson (1928), Levinthal (1926), and others). The Griffith method of conversion from the R to the S form by inoculating the R culture subcutaneously into mice with a vaccine prepared from a virulent pneumococcus, has been successfully repeated by Neufeld and Levinthal (1928), Reimann (1929), and Dawson (1930).

The change of the virulent to the avirulent form has been more easily accomplished than the reverse process. This has been done by growing the organisms in plain broth (Eyre and Washburn (1897)), Cotoni (1912), and Wadsworth and Kirkbride (1918); by repeatedly growing them in the presence of type specific anti-serum (Stryker (1916), Griffith (1923)) or in the presence of bile (Reimann (1925)).

The mechanism by means of which the pneumococcus exerts pathogenicity is still open to discussion. The soluble specific substance, isolated first by Dochez and Avery (1917) and purified by Heidelberger and Avery (1924) and Heidelberger and Goebel (1926) according to the claims of Felton and Bailey (1926) increases the virulence of virulent pneumococci and neutralizes the protective substances of the anti-pneumococcus serum. The latter observation was made earlier by Cole (1917). The search

for a soluble toxin by many workers has not been fruitful. Among these may be mentioned Kruse and Pansini (1892), MacFadyen (1906), Wadsworth (1912), and Chesney and Hodges (1922). Recently a potent filtrate was reported by Olson (1925-1926). The details of this work have not been published, nor has there been further report on this toxin. Cole (1914), Weiss and Kolmer (1918), and others demonstrated that when pneumococci were dissolved in sodium choleate this solution was toxic for laboratory animals in amounts of several cubic centimeters. Cole (1912) also found that saline extracts were sometimes toxic and Neufeld and Dold (1911) demonstrated that extracts made with salt solution which contained 0.1 per cent lecithin were also toxic. Furthermore, saline extracts of pneumonic lungs have been found to be toxic by Rosenow and Arkin (1912), Weiss, Kolmer and Steinfield (1918), and Solis-Cohen, Weiss and Kolmer (1918). An extract of the pneumococcus was demonstrated by Rosenow (1907) to have the capacity of decreasing the phagocytosis of avirulent organisms and he also claimed that when avirulent organisms were incubated in this extract for twenty-four hours they became virulent for guinea pigs. Julianelle and Reimann (1926) and later Mair (1928) have reported the production of purpura in white laboratory animals following the inoculation of an extract produced by freezing and thawing the pneumococcus but this substance was not lethal unless it were given in large amounts.

The skin reactions produced by Zinsser and Grinnell (1927) and Bull and McKee (1929) indicate that autolysates probably play a rôle in pneumococcus infections. The anaerobic autolysate prepared recently by Parker (1928) and Parker and Pappenheimer (1928) is the most toxic pneumococcal product that has been reported.

The study of the blood in pneumococcus pneumonia, made by Bieganski (1894), Bentz (1918), Tongs (1922), Pons and Ward (1925) and Haden (1925), lend evidence to the view that a toxic substance is liberated. One effect produced on the blood by the purpura-producing extract is similar to that observed in patients with lobar pneumonia: the reduction in the number of the blood platelets (Reimann (1924), and Reimann and Julianelle (1926)).

Of interest is the claim of Prati and Cavazzuti (1925) that the mere presence of pneumococci in the lungs does not entail pneumonia but that the addition of autolytic products from the lungs or other tissues causes a fatal inflammation.

In the hands of the majority of workers the best results in the production of experimental pneumococcus lobar pneumonia have been produced when the host was, to some extent, resistant to the organisms. Among these workers should be mentioned Wadsworth (1904), Blake and Cecil (1920), Permar (1923), Stillman and Branch (1924), and Nakajima (1926). To increase the resistance of the host the majority of the workers administered a vaccine. Wadsworth partially immunized a few rabbits with filtrates. The theory that the type of lesion produced experimentally is due to the virulence of the organism has been stressed by Lamar and Meltzer (1912), Gaskell (1925, 1928), Armstrong and Gaskell (1921) and Whittle (1928).

In the work with different species of animal, different types of pneumonic lesions have been observed. These are probably due to the difference in the anatomy of the lung and Schöbl and Sel-lards (1926), and Whittle think that there is no fundamental difference between pneumonia in man and artificial pneumonia in animals.

The study reported in this paper includes observations on the influence of saline extracts, of purpura-producing extracts, of substances that lower and raise the electrophoretic potential of negatively charged membranes or surfaces, and of filtrates on the virulence of the pneumococcus; and observations on the influence of partial immunization on the course of pneumococcus infections in rabbits.

#### GENERAL METHODS

The work with the pneumococcus has been done with a Type I strain and four of its variants. Three of these cultures designated as PnA orig., PnB orig. and PnC orig. were secured from Dr. F. G. Blake in 1924. Their virulences for white mice are, respectively,  $1 \times 10^{-8}$  cc.,  $1 \times 10^{-2}$  to  $1 \times 10^{-3}$  cc., and 1.0 cc. or more. An avirulent variant, designated as A23R or A26R, was

derived from the PnA culture by transferring it 23 to 26 times in plain broth containing 10 per cent Type I anti-pneumococcus serum. The fifth culture varied from PnA orig. in its ability to kill rabbits in high dilutions. This property had been developed by transferring the culture through a number of rabbits.

A culture of *B. paratyphosus* B designated as 355R was obtained from Dr. Jordan's (1926) laboratory. This culture is of the Schottmüller type and is avirulent for mice and guinea pigs.

The results of virulence measurements of the cultures are expressed in terms of minimal lethal dose (M.L.D.) in cubic centimeters for white mice. The methods of preparation of the culture for the determination of the M.L.D. have been described by Falk, Gussin and Jacobson (1925). The procedure was as follows:

- (1) Twenty-four-hour culture on blood agar slant + 10 cc. buffered broth. Incubate for five hours.
- (2) 0.5 cc. broth suspension + 10 cc. buffered broth incubate sixteen to eighteen hours at 37°C.
- (3) Prepare decimal dilutions of culture in sterile broth so that quantities of the culture from 0.1 to 0.000,000,01 cc. are contained in a volume of 0.5 cc.
- (4) Inject 0.5 cc. of dilution into mice intraperitoneally.

The specificity of the death of each animal under observation was tested by taking cultures from the heart's blood and attempting to recover the pneumococcus. Peripheral blood cultures were made from mice by sterilizing the tail with alcohol, drying with ether and clipping a small portion from it with sterile scissors. All inoculations into mice were made intraperitoneally, unless otherwise stated.

The agglutination tests were made with organisms grown in 0.5 per cent glucose broth. To 0.3 cc. of the culture was added 0.3 cc. of an 0.85 per cent NaCl dilution of Type I anti-pneumococcus serum which had been obtained from the New York State Department of Health. The culture-serum mixtures were held for one hour in a water bath adjusted to 38°C. and in the ice-box over night. Two readings were made.



The apparatus and procedure for the measurement of the electrophoretic potentials have been described by Falk and Jacobson (1925).

Wright's technique has been used for the phagocytosis experiments. The average number of bacteria per leucocyte was determined by counting the bacteria in one hundred cells.

*Media.* 1. The blood agar used for slants and plates consisted of 2 per cent agar, veal infusion nutrient broth, and 8 to 10 per cent defibrinated sheep's blood.

2. The blood broth consisted of veal infusion peptone broth containing eight to ten parts of defibrinated sheep's blood.

3. The buffered veal infusion broth contained 1 per cent peptone and 0.2 per cent sodium phosphate.

#### EXPERIMENTAL FINDINGS

##### *I. The influence of "virulin" on avirulent pneumococci*

In 1907 Rosenow reported that a saline extract of pneumococci, "virulin," decreases the phagocytosis of avirulent pneumococci and that when the avirulent organisms are incubated with the extract they become virulent for guinea pigs. This paper has been referred to frequently in textbooks and journal reports, but we are unaware of any publication that describes its repetition.

1. *The effect of "virulin" on the virulence of avirulent organisms for mice.* The method of extracting the pneumococci was similar to that used by Rosenow. It is as follows: large quantities of the organisms were suspended in small amounts of 0.85 per cent NaCl and incubated at 37°C. for forty-eight hours, heated at 60°C. for one hour and then centrifugalized to remove the detritus. Extracts were made from virulent and avirulent cultures, PnA orig. and A23R.

The organisms were prepared by the usual technique for virulence except that when they were five hours old they were added to an equal quantity of "virulin." Rosenow washed the organisms before he added the "virulin," but we found that if no broth was present there was a decline in the number of organisms.

Although there were a number of nutritive substances in the extract, it was by itself deficient in maintaining the viability of all the organisms. After the mixture had been incubated for twenty-four hours it was inoculated into mice. Control animals were inoculated with organisms that had been incubated for the same length of time in the avirulent pneumococcus extract, and in saline. All animals survived the largest dose, 1.0 cc.

Before the mice were inoculated streaks from the culture were made on blood agar for colony observation. No change in colony appearance could be detected.

TABLE 1

*The influence of "virulin" on phagocytosis of avirulent pneumococci*

STRAIN	SERUM	LEUCOCYTES	BACTERIA PER PHAGOCYTE*
A23R ("virulin")†.....	Normal rabbit	Rabbit	4 7
A23R (A23R ex.).....	Normal rabbit	Rabbit	9 0
A23R (NaCl).....	Normal rabbit	Rabbit	11 4
A23R ("virulin")‡.....	Immune horse	Rabbit	5 3
A23R ("virulin").....	Immune horse	Rabbit	7 9
A23R (NaCl).....	Immune horse	Rabbit	12 0
PnC ("virulin").....	Immune horse	Rabbit	2 6
PnC (NaCl).....	Immune horse	Rabbit	7 7

\* Average number of bacteria in 100 leucocytes.

† Substances in parentheses indicate those in which the organisms had been incubated.

‡ Unwashed organisms.

2. *The influence of "virulin" on phagocytosis of avirulent pneumococci.* Avirulent organisms were grown for twenty-four hours in buffered broth, centrifugalized, and resuspended in a small amount of the supernatant fluid. This heavy suspension was divided into three parts. Then equal parts of "virulin," avirulent extract and saline were added. After the mixtures were incubated for twenty-four hours and washed they were used for the phagocytosis test. Rabbit leucocytes, normal rabbit serum and immune horse serum were used. The time of incubation was twenty minutes.

The results tabulated in table 1 show that there was more than a 50 per cent decrease in the phagocytosis of the organisms that had been incubated with the "virulin" while there was a slight decrease due to the avirulent extract. This experiment was repeated several times, but at no time was the decrease as marked as Rosenow reported.

3. *The influence of "virulin" on opsonic serum.* To observe the influence of the extract on the opsonin content of the serum, equal parts of the extract or saline were added to sera. Both normal and immune rabbit sera were used. The mixtures were incu-

TABLE 2  
*The influence of "virulin" on opsonic serum*

CULTURE	EXTRACT OF	RABBIT SERUM	BACTERIA PER PHAGOCYTE
PnA orig.	PnA orig.	Normal	0.3
	PnA orig.	Immune	0.2
	A23R	Normal	0.5
	A23R	Immune	1.0
	NaCl	Normal	0.68
	NaCl	Immune	0.5
A23R	PnA orig.	Normal	1.6
	PnA orig.	Immune	3.2
	A23R	Normal	4.4
	A23R	Immune	6.7
	NaCl	Normal	3.9
	NaCl	Immune	6.9

bated in a water bath at 37°C. for one hour. Then the phagocytosis tests were made by adding an equal volume of a suspension of washed rabbit leucocytes and of bacterial culture. Virulent and avirulent cultures were used.

Again, the number of pneumococci phagocytosed was greatest from the saline control tube and least from the "virulin" tube. The difference in the phagocytosis of virulent and avirulent organisms was also observed.

This experiment was repeated with normal and immune horse sera instead of rabbit sera. The results were in harmony with the preceding experiment, the greatest decrease in virulence

being observed when the pneumococci had been incubated in the presence of "virulin."

Mice were inoculated with 2 cc. of the serum-extract-leucocyte-bacterium mixture after it had been incubated for twenty minutes. A23R and PnC orig. cultures were used. The mice suffered no ill effects.

4. *The rapid transfer of cultures in "virulin."* PnB orig. and PnC orig. were seeded in a medium which contained equal parts of buffered broth and extract. An extract of an avirulent culture and saline added to the broth were used as control media. Transfers were made twice daily and virulence tests were made after 4, 13, 26 and 49 transfers. There seemed to be a slight increase in the virulence of PnB after the transfers in both extracts. But this increase was as noticeable after the fourth transfer as it was at the forty-ninth. Table 3 is given for the comparison of the virulence of PnB after six transfers in the three media. It will be noted that the mouse which received the lowest dilution of the culture grown in the presence of "virulin" failed to die, although it developed a severe septicemia; while the other mice which received the culture up to a dilution of  $1 \times 10^{-4}$  died. The size of the mouse should be mentioned as a possible explanation of the survival as it was larger than the other mice. The culture grown in the avirulent extract was lethal in a dilution of  $1 \times 10^{-2}$ , while the control culture grown in the presence of NaCl failed to kill in the lowest dilution used ( $1 \times 10^{-1}$ ). The organisms from the saline control tube had decreased in virulence, and it is thought that there was a slight increase in the virulence of the organisms from the tube which contained "virulin."

The difference in virulence of PnC orig. was less marked than with PnB orig. This experiment suggests that "virulin" tends slightly to increase the virulence of a pneumococcus of medium virulence (or at least the virulence of the organism is maintained) while it has little influence on one of lesser virulence.

*Discussion.* It is apparent from this study that "virulin" definitely decreases the phagocytosis of avirulent pneumococci. The decrease was never very marked, but it was always consistent. The extract of avirulent organisms also causes a slight decrease

TABLE 3  
Experiment 14. The effect of rapid transfer of PnB in the presence of "virulin"

MOUSE NUMBER	CULTURE*	EXTRACT OF	AMOUNT OF CULTURE cc.	DAILY BLOOD CULTURES AND RESULTS								
				1	2	3	4	5	6	7	8	9
31	PnB(26)	PnA orig.	10 <sup>-1</sup>	++	+++	+	+	+	-	-	-	-
31'	PnB(26)	PnA orig.	10 <sup>-2</sup>	D†	+	D	-	-	-	-	-	-
32	PnB(26)	PnA orig.	10 <sup>-3</sup>	+	+	-	-	-	-	-	-	-
32'	PnB(26)	PnA orig.	10 <sup>-4</sup>	D†	+	-	-	-	-	-	-	-
33	PnB(26)	PnA orig.	10 <sup>-5</sup>	-	+	-	-	-	-	-	-	-
34	PnB(26)	A23R	10 <sup>-1</sup>	+++	+++	+++	-	-	-	-	-	-
34'	PnB(26)	A23R	10 <sup>-2</sup>	++	++	++	D	++	+++	++	-	D
35	PnB(26)	A23R	10 <sup>-3</sup>	+	+	+	++	+	-	-	-	-
35'	PnB(26)	A23R	10 <sup>-4</sup>	-	-	-	-	-	-	-	-	-
36	PnB(26)	A23R	10 <sup>-5</sup>	+	-	-	-	-	-	-	-	-
37	PnB(26)	NaCl	10 <sup>-1</sup>	+	+++	+++	+	+	-	-	-	-
37'	PnB(26)	NaCl	10 <sup>-2</sup>	+	++	+	-	-	-	-	-	-
38	PnB(26)	NaCl	10 <sup>-3</sup>	-	-	-	-	-	-	-	-	-
38'	PnB(26)	NaCl	10 <sup>-4</sup>	-	-	-	-	-	-	-	-	D‡
39	PnB(26)	NaCl	10 <sup>-5</sup>	-	-	-	-	-	-	-	-	-

\* Culture 6 hours old.

† D = death.

‡ Non-specific organisms from heart's blood.

§ Heart's blood sterile.

+++ , septicemia heavy; ++ , moderate; + , slight; -, none.

in the phagocytosis. Since the bacteria were washed free of the extract before the tests were made it would seem that the extract had effected some change in the organism.

The opsonin content of serum was also affected by the extract. When serum-leucocyte mixtures were incubated with the extract before the addition of the bacteria for the test there was a decrease in phagocytosis; but the decrease was not so marked as when the bacteria were incubated with the extract before the phagocytosis test. Since the extract was present during the reaction it can not be definitely concluded that the action of the extract was limited to the serum.

When avirulent pneumococcus cultures were incubated in the presence of equal amounts of the extract and then inoculated into mice, the animals suffered no ill effects. Rosenow reported that such mixtures killed guinea pigs. His cultures, which had lost their virulence while being held for a few months without animal passage, and which could be quickly restored to their original virulence by animal passage, were not as potentially avirulent as the culture used in our experiments. When a slightly virulent culture was transferred rapidly in broth containing some of the extract, the virulence remained constant or was perhaps slightly increased, while the culture in the control medium of broth and saline lost in virulence.

Since guinea pigs can be killed with large amounts of saline extracts (Cole (1912)) it is possible that Rosenow used a more concentrated extract (although we attempted to follow his technique) and that death was largely due to the extract and not to the increase in virulence of the pneumococcus.

Our results have confirmed Rosenow's work so far as it pertains to the decrease of phagocytosis of avirulent pneumococci with "virulin," but we were unable to demonstrate that "virulin" causes an avirulent pneumococcus to become virulent for mice.

## *II. The influence of freezing and thawing extract on the virulence of pneumococci*

After it was found that the saline extract gave very feeble results in enhancing virulence, attention was directed toward another extract prepared by breaking up the organisms.

*Preparation of the extract.* The bacteria were grown for sixteen hours in glucose broth, then concentrated to  $\frac{1}{40}$  of the original volume. At first the organisms were suspended in saline and later they were allowed to remain in a small amount of the supernatant fluid. For freezing, about 15 cc. of the heavy suspension were placed in a 40 cc. ordinary glass centrifuge tube which contained a short piece of rubber tubing closed at each end with a rubber cap. The organisms were frozen in liquid air and thawed in a 37°C. water bath. The process of freezing and thawing was continued until the breaking of the cells was complete. Ten freezings were usually sufficient for the avirulent pneumococci, but more were necessary for the virulent organisms. The detritus was removed by centrifugation and the supernatant, which was generally sterile, was stored in the ice-box until used. If after culturing, it was found to contain a few viable pneumococci, it was filtered through a Berkefeld V filter. An extract of A23R was used for most of the experiments.

From thirty minutes to several hours after the intraperitoneal inoculation of the extract into mice they developed purpuric spots on one or all of the following parts of the body: tail, ears, feet, nose and genitalia. The reaction reached its maximum in twenty-four hours and persisted from four to seven days. Table 5 illustrates the time of development of the reaction after the inoculation. The duration is recorded in tables 4, 6, and 7. A few white guinea pigs were inoculated intracutaneously. These animals also developed purpura and at the site of inoculation there was a white area surrounded by an inflammatory reaction which persisted for three days. It then gradually disappeared and was followed by desquamation.

During the following experiments autopsy records of the mice were kept. This study is reported in the following paper (Pittman and Southwick).

1. *The influence of two-hour intervals between inoculations of extract and varying amounts of pneumococci.* During some preliminary experiments with mice there were indications that this extract might change the course of an infection with a pneumococcus culture. To observe this more closely mice were inoculated in-

traperitoneally with 0.25 cc. of the extract two hours preceding, simultaneously, and two hours following, the intraperitoneal inoculation of varying amounts of cultures of PnC orig. and PnB orig. The mice which received the extract preceding and simultaneously with the culture PnC orig. did not develop any blood infection and only one of the control mice which received saline instead of the extract developed a very slight blood infection, present for only one day. All the mice that received the extract following the culture on the other hand, developed a heavy septicemia which terminated in death. It was also noted that the purpuric reaction was not so severe when the extract was given simultaneously with, or following, the culture.

The differences with PnB orig. were not so striking as with PnC orig. A possible explanation of the difference may lie in the virulence of the organism. PnC orig. is a borderline organism while PnB orig. is a thousand times more virulent for mice.

To observe further the influence of the extract, the extract was given six hours preceding the cultures.

2. *The influence of extract on infection when the extract precedes the culture by six hours.* In this experiment graded amounts of the extract 0.125 to 1.0 cc. were given six hours preceding the inoculation of a constant amount, 0.5 cc., of the culture PnC orig. At the time when the culture inoculations were being made seven hitherto untreated mice were inoculated with the culture. Eighteen hours afterward blood cultures were made and six of the mice that had not received the extract were given graded amounts of the extract.

Of the 5 mice that received the extract preceding the culture only 1 died. At the time of the culture inoculation this mouse, no. 7, was subnormal in temperature and cyanotic. At autopsy five large cysticercoïds were found on the liver and the heart's blood culture gave only one colony of pneumococci. On the second day mouse 5 showed a mild septicemia.

Before the control animals received the extract, 4 had a mild septicemia and the blood culture of another was questionable. Four of the 6 mice that received the extract died and the culture control animal also died. It was also noted that the purpuric



TABLE 4  
Experiment 2. The influence of 2-hour intervals between inoculations of extract and pneumococci

MOTHS NUM- BER	CULTURE*	AMOUNT AMOUNT	EXTRACT AMOUNT	TIME INTERVAL	FURFURA DAILY					DAILY BLOOD CULTURES									
					1	2	3	4	5	1	2	3	4	5	6	7	8	9	0
1	PnC orig.	cc.	cc.	-2 hours	+	++	+	+	-	-	-	-	-	-	-	-	-	-	-
1'	PnC orig.	1.5	0.25†	-2 hours	+	++	++	+	-	-	-	-	-	-	-	-	-	-	-
2	PnC orig.	1.0	0.25†	-2 hours	+	++	++	+	-	-	-	-	-	-	-	-	-	-	-
2'	PnC orig.	0.5	0.25†	-2 hours	+	++	++	+	-	-	-	-	-	-	-	-	-	-	-
3	PnC orig.	1.5	0.25†	Simultaneously	-	++	++	+	-	D†	-	-	-	-	-	-	-	-	-
3'	PnC orig.	1.0	0.25†	Simultaneously	+	++	+	-	-	-	-	-	-	-	-	-	-	-	-
4	PnC orig.	0.5	0.25†	Simultaneously	+	++	+	-	-	-	-	-	-	-	-	-	-	-	-
4'	PnC orig.	1.5	0.25†	+2 hours	+	++	+	-	-	-	-	-	-	-	-	-	-	-	-
5	PnC orig.	1.0	0.25†	+2 hours	+	++	+	-	-	-	-	-	-	-	-	-	-	-	-
5'	PnC orig.	0.5	0.25†	+2 hours	+	++	+	-	-	-	-	-	-	-	-	-	-	-	-
6	PnC orig.	1.5	NaCl	Simultaneously	-	-	-	-	-	+	++	++	++	++	D	-	-	-	-
6'	PnC orig.	1.0	NaCl	Simultaneously	-	-	-	-	-	+	++	++	++	++	-	-	-	-	-
	PnC orig.	0.5	NaCl	Simultaneously	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	PnB orig.	10 <sup>-1</sup>	0.25	-2 hours	++	++	+	-	-	-	-	-	-	-	-	-	-	-	-
7'	PnB orig.	10 <sup>-2</sup>	0.25	-2 hours	+	+	±	-	-	++	++	++	++	++	+	D†	-	-	-
8	PnB orig.	10 <sup>-4</sup>	0.25	-2 hours	++	++	++	+	±	++	++	++	++	++	++	+	-	-	-
8'	PnB orig.	10 <sup>-2</sup>	0.25	Simultaneously	+	+	±	-	±	++	++	++	++	++	++	+	-	-	-
9	PnB orig.	10 <sup>-1</sup>	0.25	Simultaneously	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
9'	PnB orig.	10 <sup>-4</sup>	0.25	Simultaneously	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
10	PnB orig.	10 <sup>-1</sup>	0.25	+2 hours	++	++	++	++	±	++	++	++	++	++	++	++	++	++	++
10'	PnB orig.	10 <sup>-2</sup>	0.25	+2 hours	++	++	++	++	-	++	++	++	++	++	++	++	++	++	++
11	PnB orig.	10 <sup>-4</sup>	0.25	+2 hours	++	++	++	++	±	++	++	++	++	++	++	++	++	++	++
11'	PnB orig.	10 <sup>-2</sup>	NaCl	Simultaneously	++	++	++	++	-	++	++	++	++	++	++	++	++	++	++
12	PnB orig.	10 <sup>-1</sup>	NaCl	Simultaneously	-	-	-	-	-	++	++	++	++	++	++	++	++	++	++
12'	PnB orig.	10 <sup>-4</sup>	NaCl	Simultaneously	-	-	-	-	-	++	++	++	++	++	++	++	++	++	++

\* Culture 12 hours old.

† 0.25 extract + 0.25 cc. NaCl.

‡ Heart's blood sterile.

TABLE 5  
*Experiment 3. The influence of extract on infection with PnC orig.*

MOUSE NUMBER	AMOUNT OF EXTRACT	EXTRACT INOCULATION IN RELATION TO CULTURE INOCULATION	AMOUNT OF CULTURE	PRESENCE OF PURPURA				DAILY BLOOD CULTURES								
				3½ hours	4 hours	6½ hours	24 hours	18 hours	2 days	3	4	5	6	7	8	
	cc.		cc.													
5	0.125	6½ hours before	0.5	+	++	++	++	-	+	-	-	-				
6	0.25	6½ hours before	0.5	-	+	++	++	-	-	-	-	-				
7	0.375	6½ hours before	0.5	±	+	++*	++	D	-	-	-	-				
8	0.5	6½ hours before	0.5	-	++	+++	+++	-	-	-	-	-				
9	1.0	6½ hours before	0.5	+	+	++	+++	-	-	-	-	-				
11	0.03	18 hours after	0.5	-	-	-	-	+	-	-	-	-				
11'	0.06	18 hours after	0.5	-	-	-	-	++	++	D	+	++				
12	0.125	18 hours after	0.5	-	±	±	+	++	++	++	++	++		D		
12'	0.25	18 hours after	0.5	-	-	-	-	-	+	-	-	-				
13	0.5	18 hours after	0.5	-	±	±	+	+	++	D	+	D†				
13'	1.0	18 hours after	0.5	-	-	-	-	?	+	+	+	+				
14	-	18 hours after	0.5	-	-	-	-	-	+	+	+	+		+		D
5'	0.125	8 days before	0.5	-	+	++	+++	-	-	-	-	-				
6'	0.25	8 days before	0.5	-	+	++	+++	-	+	-	-	-				
7'	0.375	8 days before	0.5	-	-	+	++	-	+	++	++	++		D		
8'	0.5	8 days before	0.5	-	+	++	+++	-	D	+						
9'	1.0	8 days before	0.5	+	++	+++	+++	-	-	-	-	-				

\* Temperature subnormal.

† Heart's blood sterile.

reaction was absent or less severe in the animals that had received the culture preceding the inoculation of the extract.

The 5 mice that received the extract and no culture were inoculated with PnC orig. culture eight days after the extract. At this time all signs of purpura had disappeared. Three of these mice developed a septicemia and 2 died.

The experiment when repeated gave the same results, namely, that (1) extract in amounts of 0.5 cc. or more when given six hours preceding the culture usually protected the mice against infection; that (2) this protection did not last as long as eight days; that (3) death resulted when extract was given after a slight infection had developed; and that (4) when the extract followed the culture inoculation the purpuric reaction was absent or less severe.

3. *The influence of extract on infection when the extract precedes the culture from seven days to two hours.* To determine more accurately how long the extract protected the mouse against an infection with PnC orig., a series of duplicate mice were inoculated with extract from seven days to two hours preceding the culture inoculation. All the mice that received the extract preceding the culture inoculation developed purpura, but the purpura on mice that had received the extract seven days previous to the culture had been blanched for three days.

In table 6 it appears that all mice that had had the extract more than one day were protected against infection if the purpuric reaction was present. The protection varied with those mice which received the extract two to twenty-four hours preceding the culture, but none died which received the extract more than eight hours before the culture. The two control mice each developed a septicemia and one died.

4. *Further observations on the protective action of the extract.* This time the mice were inoculated with the extract as in the preceding experiment but in triplicate instead of duplicate. The third mouse was added to the series in order that one might be killed at the time the others were inoculated and its tissues be examined microscopically. In each group one mouse received 0.5 cc. of the culture prepared by the standard technique for

virulence, but the other mouse received twice the number of organisms after they had been concentrated to one-half the original volume.

TABLE 6

*Experiment 6. The influence of extract on infection when the extract precedes the culture\* from seven days to two hours*

MOUSE NUMBER	AMOUNT OF EXTRACT	TIME INTERVAL	PURPURIC REACTION		DAILY BLOOD CULTURES								
			Sever- ity	Presence	1	2	3	4	5	6	7	8	9
1	0.5	7 days	++	Absent†	+	+++	D						
1'	0.5	7 days	++	Absent	+	++	-	+	+	+			D
2	0.5	5 days	+++	Present	-	-	-	-	-				
2'	0.5	5 days	++	Present	-	-	-	-	-				
3	0.5	3 days	+++	Present	-	-	-	-	-				
3'	0.5	3 days	++	Present	-	-	-	-	-				
4	0.5	2 days	+	Present	-	-	-	-	-				
4'	0.5	2 days	++	Present	-	-	-	-	-				
5	0.5	24 hours	+	Present	-	-	-	-	-				
5'	0.5	24 hours	+++	Present	+	++	+	+	-	+			D‡
6	0.5	18 hours	++	Present	-	-	-	-	-				
6'	0.5	18 hours	+++	Present	-	-	+	+	-	-			
7	0.5	8 hours	++	Present	-	+++	+++	D					
7'	0.5	8 hours	+++	Present	+++	+++	D						
8	0.5	4 hours	+++	Present	+	++	+	-	-	-			D‡
9	0.5	2 hours	++	Present	-	-	-	-					
9'	0.5	2 hours	++	?	-	-	+++	D					
10	0.5	0	+	-	+++	+++	D						
10'	0.5	0	+	-	+++	+++	+++	++	++	++	D		
11	-		-		+	+	-		-	-			
11'	-		-		++	+++	+++	D					

\* Each mouse received 0.5 cc. of PnC orig. culture.

† Purpura had been blanched for 3 days.

‡ Heart's blood sterile.

On inspection of table 7 it will be observed again that if the mice had had the extract more than one day and if purpura was still present, they were generally protected against infection. The control animals developed a septicemia and the one that received the concentrated culture died.

PnC orig. was transferred twice daily for 47 transfers in a

TABLE 7  
Experiment 8. Repetition of experiment 6 with the culture concentrated

MOUSE NUMBER	AMOUNT OF EXTRACT	TIME INTERVAL	PURPURIC REACTION		AMOUNT PnC CULTURE	DAILY BLOOD CULTURES						
			Severity	Presence		1	2	3	4	5	6	9
	cc.				cc.							
17	0.5	7 days	++++	Blanching	K*	-	-	+++	+++	D		
17'	0.5	7 days	++++	Absent	0.5C†	-	-	+++	+++			
18	0.5	5 days	++++	Blanching	K	-	-	+++	D	-		
18'	0.5	5 days	+++	Absent	0.5C	-	-	-	-	-		
19	0.5	3 days	+	Present	0.5C	-	-	-	-	-		
19'	0.5	3 days	+++	Present	0.5	-	-	-	-	-		
19"	0.5	3 days	+++	Present	K	-	-	-	-	-		
20	0.5	2 days	++++	Present	0.5C	-	-	-	-	-		
20'	0.5	2 days	+++	Present	0.5	-	-	-	-	-		
20"	0.5	2 days	+++	Present	K	-	-	-	-	-		
21	0.5	24 hours	++++	Present	0.5C	-	++	++	++	++	D	
21'	0.5	24 hours	++++	Present	0.5	-	-	-	-	-		
21"	0.5	24 hours	++++	Present	K	-	-	-	-	-		
22	0.5	18 hours	++++	Present	0.5C	-	-	-	-	-		
22'	0.5	18 hours	++++	Present	0.5	-	-	-	-	-		
22"	0.5	18 hours	++++	Present	K	-	-	-	-	-		
23	0.5	8 hours	+	+	0.5C	+	+++	++	++	++	D	
23'	0.5	8 hours	++++	++	0.5	-	-	-	-	-		
23"	0.5	8 hours	++++	++	K	-	-	-	-	-		
24	0.5	4 hours	++++	++	0.5C	-	-	+	+	-		
24'	0.5	4 hours	++++	++	0.5	+++	+++	D	-	-		
24"	0.5	4 hours	++	+	K	+++	+++					
25	0.5	2 hours	-	-	K	++	++	D				
25'	0.5	0	++++	-	0.5C	++	++	D				
25"	0.5	0	++++	-	0.5	+++	+++	D				
26	-		-		0.5C	+++	+++	D				
26'	-		-		0.5	-	±	+	-			

\* K = killed with chloroform vapor when other mice were inoculated with culture.

† C = culture twice concentrated.

medium which consisted of one part of extract and seven parts of broth. Three virulence tests were made and frequent observations were made of the colony appearance. No change in the organism could be detected.

*Discussion.* The extract prepared by repeatedly freezing and thawing pneumococci which has been used in the preceding experiments produced gross lesions in the mouse that seem to be identical with the lesions described by Julianelle and Reimann (1926). It was also found that both virulent and avirulent pneumococci gave the purpura-producing substance, but that the virulent organisms required more freezing and thawing to break the cell walls than did the avirulent pneumococci.

The limited scope of this paper will not allow us to draw any definite conclusions as to the nature of the purpura-producing substance. Julianelle and Reimann have held that it is a degradation product of the pneumococcus and Mair that it exists preformed in the cell and that dissolution is all that is necessary to elicit the principle.

This extract seems to increase the virulence of PnC orig., a culture of very low virulence, when it is given simultaneously with the culture or when it is given after a slight septicemia has developed. Two suggestions may be offered to explain this increase in virulence. Since the extract is slightly toxic for the mouse it may be that the extract lowers its resistance sufficiently to allow the invasion of the organisms, or the substance may act as an aggressin and prevent the phagocytosis of the organism.

As opposed to the increase in virulence thus obtained, was the protection, not only against death but against blood invasion, that the extract afforded the mouse if it were given twenty-four hours preceding the culture inoculation (sometimes this protection was noted if the extract preceded the infection by two hours). As long as the purpura was visible the mouse was protected against at least twice the inoculum that usually caused a slight septicemia. The control animals which received this concentrated culture died. A more concentrated culture was not tried to test the limit of the protection against the borderline culture PnC orig. When a more virulent culture was used the protection

was inconstant, but cultures that are moderately virulent for mice usually increase in virulence *in vivo* much more rapidly than borderline cultures. The presence of antibodies against the pneumococcus can not be the cause of the protection as the mice were not protected after the purpura blanché. Furthermore,

TABLE 8

*Experiment 7. Influence of filtrates on the virulence of PnC orig.*

MOUSE NUMBER	FILTRATE OF	BROTH CONTAINED	AMOUNT OF FILTRATE	AMOUNT OF CULTURE	DAILY BLOOD CULTURES					
					1	2	3	4	5	6
			cc.	cc.						
16	PnC	Ce(NO <sub>3</sub> ) <sub>2</sub>	2 0	—	—	K*				
16'	PnC	Ce(NO <sub>3</sub> ) <sub>2</sub>	1 0	0.5	+++	+++	+++	+++		D
15	PnC	La(NO <sub>3</sub> ) <sub>2</sub>	2.0	—	—	K				
15'	PnC	La(NO <sub>3</sub> ) <sub>2</sub>	1.0	0.5	D					
14	PnC	0.5 per cent Glu.	2 0	—	—	K				
14'	PnC	0.5 per cent Glu.	1.0	0.5	+++	D				
13	PnC	Glu. + Ser.	2.0	—	—	K				
13'	PnC	Glu. + Ser.	1.0	0.5	+++	D				
12	PnC	—	2.0	—	—	K				
12'	PnC	—	1.0	0.5	D					
11	—	—	—	0.5	+	+	K+			
6	PnA	Ce(NO <sub>3</sub> ) <sub>2</sub>	2.0	—	—	K				
6'	PnA	Ce(NO <sub>3</sub> ) <sub>2</sub>	1.0	0.5	—	—				
7	PnA	La(NO <sub>3</sub> ) <sub>2</sub>	2.0	—	—	K				
7'	PnA	La(NO <sub>3</sub> ) <sub>2</sub>	1.0	0.5	+++	D				
8	PnA	0.5 per cent Glu.	2.0	—	—	K				
8'	PnA	0.5 per cent Glu.	1.0	0.5	+++	+++	+++			D
9	PnA	Glu. + Ser.	2 0	—	—	K				
9'	PnA	Glu. + Ser.	1.0	0.5	+++	+++	D			
10	PnA	—	2.0	—	—	K				
10'	PnA	—	1.0	0.5	—	—	—			
11'	—	—	—	0.5	—	—	—			

\* Killed with chloroform.

Julianelle and Reimann have demonstrated that the extract is non-antigenic. The influence of the extract on the leucocytes removes the possibility that there has been a leucocytic stimulation in the blood. Reimann and Julianelle noted that the leucocytes were but slightly if at all influenced by the extract. Dr.

G. W. Stuppy and we ourselves have noted in monkeys treated with extract that there was a slight reduction in leucocytes. Regeneration was accomplished by the third day, but there was no over-regeneration. The destruction of the erythrocytes and platelets may cause the liberation of a substance that is antagonistic to the bacteria. It should be mentioned that the extract may cause such a reaction as is noted when non-specific proteins are used therapeutically. Sufficient observations have not been made to draw an analogy between the extract reaction and non-specific therapy.

It has been observed that the purpura reaction is less severe, or absent if the mouse has received the culture a short time before the inoculation of the extract. This suggests that the bacteria exert some influence on the prevention of purpura and that the presence of the bacteria may prevent the appearance of purpura which is so rarely seen in pneumococcus infections in man. Can this be a partial answer to the question, why there is no purpura following crisis in pneumonia? In a study of the blood cells of man, rabbits and mice with pneumococcus infections Reimann, and Reimann and Julianelle found that there was a reduction in the number of platelets, yet there was no visible purpura. This would indicate that the purpura reaction is dependent upon something more than reduction in platelets.

### *III. The influence of filtrate on the virulence of pneumococci*

In an attempt to find a soluble toxin comparable to that reported by Olson a number of different kinds of media have been used for the production of filtrates. The media included glucose broth, serum broth, blood broth and broth with cerium or lanthanum salts; and the cultures were grown for varying periods of time. Only once did we secure a filtrate which killed white mice. This filtrate was made from a pneumococcus culture grown in serum glucose broth. The mouse developed external purpura which indicated that there had been some autolysis of the pneumococci.

Ten filtrates were prepared with the same medium, but only this one was toxic enough to kill mice. When the filtrates alone gave negative results, experiments were planned to observe the influence of the filtrates on pneumococci.



In the following experiments the filtrates were prepared from eighteen- to twenty-hour cultures and used immediately unless otherwise indicated.

1. *The influence of filtrates on the virulence of PnC orig.* Filtrates were prepared from a virulent and an avirulent culture. Five different media were used. The buffered broth was used for the foundation of the media and then the various substances noted in table 8 were added. The salts were added in the concentration of 0.00025 gram per cubic centimeter of broth. Mice were inoculated with 1 or 2 cc. The mice which received the smallest amount were inoculated simultaneously with 0.5 cc. of the PnC orig. prepared by the usual technique for virulence. The mice which received the 2 cc. quantity were killed after forty-eight hours and the tissues were examined microscopically. All except one of the mice which received the filtrates and culture died while the control mice lived. It should be mentioned, here as well as later, that the greater number of the mice that died had a pleuritis.

2. *The influence of filtrates on the virulence of PnC orig. when given at various time intervals.* Mice were inoculated with varying amounts of the filtrate two hours before, simultaneously, or two hours after, the inoculation of 0.5 cc. of PnC orig. culture. Two routes of injection, intraperitoneal and intravenous, were used for the culture and the filtrate.

In table 9 the dosages and time intervals may be observed. And it may be noted that the filtrate was only influential on the virulence of the organism when at least 1.0 cc. was given at the same time that the culture inoculation was made. The animal which received the broth control also died although the one which received the old filtrate did not develop a blood infection. The same observation was made here as in the preceding experiment, i.e., that the mouse which died after receiving the filtrate and culture developed a pleuritis while the one which died after receiving the broth and culture showed very little from the standpoint of gross pathology. Similar results were also observed in repeated experiments.

3. *The constant virulence of pneumococci when they were trans-*

ferred in filtrates containing large amounts of the soluble specific substance, in enriched media and in the presence of a few chemicals. Broth cultures of the virulent and avirulent pneumococci were incubated for eight days and then filtered. The filtrate from the

TABLE 9

*Experiment 9. Varying amounts of filtrate inoculated with PnC orig. at various time intervals*

MOUSE NUMBER	AMOUNT OF FIL- TRATE*	METHOD OF INOCU- LATION OF FIL- TRATE	TIME INTERVAL AS TO CULTURE INOCULATION	AMOUNT OF CUL- TURE	METHOD OF INOCU- LATION OF CUL- TURE	DAILY BLOOD CULTURES			
						1	2	3	4
	cc.			cc.					
28	0 5	i.p.	2 hours before	0 5	i.p.	—	—	—	—
28'	1 0	i.p.	2 hours before	0 5	i.p.	—	—	—	—
29	0 5	i.v.	2 hours before	0 5	i.v.	+	—	+	—
29'	0 5	i.p.	Simultaneously	0 5	i.p.	—	+	+	—
30	1 0	i.p.	Simultaneously	0 5	i.p.	+++	+++	D	
30'	0.5	i.p.	2 hours after	0 5	i.p.	+	—	—	—
31	1 0	i.p.	2 hours after	0.5	i.p.	—	—	—	—
31'	0 5	i.v.	2 hours after	0 5	i.v.	—	—	—	—
36'	2 0	i.p.	—	—	—	—	—	—	
	BROTH								
32	1 0	i.p.	Simultaneously	0 5	i.p.	+++	+++	D	
32'	2 0	i.p.	—	—	i.p.	—	—	—	
33	0 5	i.v.	—	—		—	—	—	
33'	—			0 5	i.p.	+	—	—	—
34	—			1 0	i.p.	—	—	—	—
34'	—			0.5	i.v.	+	—	—	—
	OLD FIL- TRATE†								
35	1 0	i.p.	Simultaneously	0.5	i.p.	—	—	—	—

\* Filtrate from a 24-hour culture of PnC orig. grown in the presence of  $\text{Ce}(\text{NO}_3)_3$ .

† Filtrate that had been stored in the ice chest 32 days.

virulent culture contained a large amount of soluble specific substances while the filtrate from the avirulent culture contained only a trace. The latter filtrate was used as a control to determine if the presence of soluble specific substances would effect the virulence of the organisms. The virulent and avirulent cul-

tures were transferred, first, in a medium which consisted of three parts of buffered broth and one part of filtrate. After the transfers had been continued for six weeks they were discontinued and the original cultures were transferred in a medium which contained an equal amount of the filtrate. During the course of the transfers, streaks of the cultures were made every few days on blood agar for colony study and bi-weekly virulence tests were made. At the termination of the transfers in the fifty-fifty mixture the electrophoretic potential and the agglutinability with type-specific antiserum of each organism was determined. The four methods of observation showed that there had been no change in virulence of either of the organisms in any media.

The two cultures used above, PnA orig. and A23R, and the borderline culture PnC orig. were transferred twice daily in broth media which was enriched with blood, glucose, glucose and serum, and on blood agar. PnC orig. was transferred rapidly in the presence of substances that lower and raise the electrophoretic potential. The substances are respectively, (1) cerium and lanthanum nitrate, (2) sodium oleate and botulinum toxin. An avirulent paratyphoid culture (355R), from which subcultures had been changed to the S form in Dr. Jordan's laboratory, was also transferred in plain broth at the time that some of the pneumococci were being transferred. Observations were made as above. The virulence of the pneumococci was not significantly effected. After PnC orig. had been transferred 112 times in cerium nitrate and in lanthanum nitrate media there seemed to be a slight increase in virulence. The paratyphoid culture changed to the S form and became virulent for mice. The time of change agreed very closely with that reported in Dr. Jordan's work.

The unsuccessful attempts to change the virulence of the pneumococci during this work are to be added to the previous failures to convert the R pneumococcus to the S form *in vitro*. When *in vitro* experiments are compared with Griffith's (1928) recent work, which was published after the completion of these experiments, it would seem that the pneumococcus, or some of its products in the presence of favorable media are necessary to change the R pneumococcus to the S form.

*Discussion.* Of filtrates produced from pneumococci grown in five different kinds of media only one caused death in a mouse, and nine other filtrates of cultures grown in the same medium were not lethal for mice. These results agree with those of preceding workers, Olson excepted, in indicating that culture filtrates of the pneumococcus are not lethal for mice.

However, when filtrates were inoculated simultaneously with the culture they tended to increase the invasive power of the borderline culture and 71 per cent of the mice which died had a fibrinopurulent pleuritis. The filtrates had no influence on the course of the infection if they preceded or followed the culture inoculation by two hours. The best results were obtained with the filtrates from cultures that had grown in the presence of cerium or lanthanum nitrate.

The slight change in the virulence of the pneumococcus may be due to some soluble product of the organisms, as the broth inoculated with the culture failed to give the same results. The histology also indicates that the filtrate produces some pathological lesions. Part of the change may be due to a different electrophoretic potential brought about by the ingredients of the medium. It was noted that when the culture was transferred rapidly in the presence of these salts there was a slight increase in virulence, but the increase was not sufficient to be of much significance.

Throughout this paper the word virulence has been used synonymously with the culture's ability to produce death. The work in this section indicates that we should here deviate from this meaning. In previous work by one of us (Falk (1928)) and confirmed in this study, it has been observed that pneumococcus cultures of low virulence have a low electrophoretic potential and cultures of high virulence have a high electrophoretic potential. It is rather significant that when the borderline culture grown in the presence of salts which lower the p.d. was inoculated, fatality was increased. But when the increase in fibrinopurulent pleuritis and peritonitis is considered it would seem that fatality was not due to the virulence of the organism when it was inoculated but to its ability to localize. Serum also

decreases the P.D. of bacteria and during an experiment to determine if there was any protective substance against the pneumococcus in normal monkey serum it was noted that the greater number of mice which succumbed showed a pleuritis whereas this condition was infrequent in the control mice.

*IV. The immunization of rabbits with pneumococcus filtrates in attempts to produce lobar pneumonia*

In the preceding section it was reported that filtrates of the pneumococcus increase the virulence of a borderline culture for mice and that filtrates alone produce slight pathological lesions. The brief work reported in this section has been done to observe the influence of immunization with filtrates on the course of pneumococcus infections in rabbits.

*Process of immunization.* A highly virulent culture was used in the preparation of the filtrates. The organisms were grown for eighteen hours in a buffered broth which contained 1 per cent sheep plasma and 0.5 per cent glucose. Then the culture was filtered through a Berkefeld N filter and the filtrate *immediately* inoculated intravenously into rabbits. The rabbits received 1 cc. of the fresh filtrate daily for ten successive days; they were allowed to "rest" for ten days; then they were inoculated intratracheally with the living organisms.

During the course of the filtrate inoculations daily temperatures were taken and the leucocytic counts were made frequently. At no time was the temperature abnormal and there was not much change in the leucocyte count, but since all gave the same general curve it is significant that there was a gradual drop in the number until the third day; then the number increased until it was slightly above normal on the tenth day. The number had returned to normal at the time the intratracheal inoculations were made. The weight of the rabbits remained constant.

A few of the rabbits were bled before the intratracheal inoculations and the protective titer of the serum was determined with mice inoculated with a virulent culture. The protection was very slight. One rabbit serum protected against a 1000 M.L.D., but the protection usually observed was effective against 100 M.L.D., only.

*Experimental findings.* A normal rabbit, no. 8, was inoculated intratracheally with 0.5 cc. of a culture of PnA which had been rapidly transferred through a number of rabbits. A few hours after the inoculation the temperature rose and remained abnormally high until the sixth day when the animal died. The leucocytic count dropped and remained low until the day of death. The observations are recorded in detail in figure 1. The macroscopical and microscopical autopsy findings are given in table 10. The diagnosis was bronchial pneumonia.

The culture from rabbit 8 was preserved and inoculated into four rabbits that had been partially immunized with filtrates and into two normal rabbits. The inoculum was 0.75 cc. of culture prepared by the standard technique for virulence tests. The observations were kept in the same manner as they were for no. 8 and the temperature and leucocyte curves are recorded in figures 1, 2, and 3. The autopsy findings appear in table 10.

All of the rabbits showed a definite rise in temperature the day following the injection and all showed a reduction in the white cells except 6 which showed a reduction only on the third day. With the exception of no. 6 the partially immunized rabbits showed a leucopenia until death, while the normal rabbits showed a slight rise the day before death. No rabbits showed a leucocytosis and fatality was 100 per cent.

The control rabbits, nos. 2 and 3, had a septicemia the day following the inoculation while those partially immunized did not show a septicemia until the day before death and only a few organisms were recovered at that time from the heart's blood at autopsy. Positive cultures were always obtained from the lung culture.

It may be observed in table 10 that practically every organ examined showed some pathological lesion. The lungs of the partially immunized rabbits showed that there had been some localization. They showed interstitial pneumonia, acute lymphangitis, hemorrhagic and inflammatory edema. One of the controls had bronchial pneumonia and the lungs of the others showed that there had been a generalized infection. Two of the three controls had severe peritonitis, while the partially immune

TABLE 10  
*Pathology observed in rabbits*

RABBIT NUMBER	PARTIALLY IM- MUNIZED	MACROSCOPICAL FINDINGS—LUNGS			MICROSCOPICAL FINDINGS—LUNGS										DIAGNOSIS
		Indurated areas	Hypereimia	Pleuritis	Bronchitis	Hemorrhage	Capillary distention	Perivascu- lar cell in- filtration	Leucocyte exudate	Edema	Fibrin	Alveolactasis	Emphy- sema	Throm- bosed blood vessels	
8		++		++	++		++		+	++	+	+	+		Bronchial pneumonia
2		+	+	+	+		++		+	++	+	+	+	+	Generalized infection
3		++	+	++	+	+	++	+	+	++	+	+	+	+	Inflammatory edema
10	+	++	+	++		+	++	+	+	++	++	++	++	++	Red hepatization (too few cells)
6	+	++	++	+		+	++	++	++	++	++	++	++	++	Acute lymphangitis and in- flammatory edema
18	+	++	++	+	+		++	++	++	++	++	++	++	+	Interstitial pneumonia and acute lymphangitis
52	+	++	++	+	+	+	+	++	++	++	++	++	++	++	Red hepatization (too few cells), lymphangitis and in- flammatory edema

RABBIT NUM- BER	MACROSCOPICAL FINDINGS				MICROSCOPICAL FINDINGS						
	Spleen	Liver	Peri- toneum	Spleen Hyperemia	Liver			Kidney	Rectus abdom- inis muscle	Heart	Peri- toneum
	En- larged	Con- gested	Peri- tonitis		Cell de- struc- tion	Con- gested	Necrosis		Waxy degen- eration	Myo- cardi- tis	Peri- tonitis
8		+	++	++		+	+	0	+	0	+
2		+	+++	+++		++	++	Spontane- ous	++	0	+
3	++	+				++	++	Acute	++	+	
10	0				++	++	+	0	+	+	
6	++		++ and thrombi	++	+	+	Foci of poly- morphs	Acute	++	+	
18	+	+	+++	++	+	++	+	Acute	++	+	
52	±	±	+	+	++	+	+	Acute	+	+	



animals showed no localization in the peritoneum. The other organs that were studied are the spleen, liver, kidney, skeletal and cardiac muscles. The spleen usually showed hyperemia and the four partially immunized rabbits showed some cell destruc-

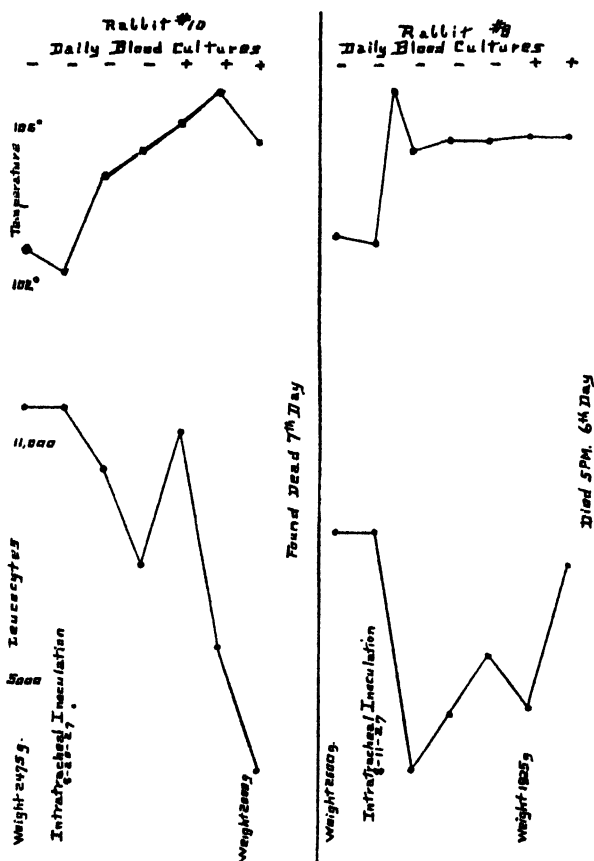


FIG. 1. EFFECT OF PNEUMOCOCCUS INFECTION IN ONE PARTIALLY IMMUNIZED AND ONE NORMAL RABBIT

tion of the spleen. The liver of each was congested and all except no. 6 showed necrotic areas. The latter had a few foci of polymorphonuclear cells. The kidneys showed a nephritis and an engorgement of the glomeruli with blood. The skeletal muscles,

abdominis rectus and diaphragm, showed Zenker's waxy degeneration. The heart muscle of the four examined showed a myocarditis. The subcutaneous blood vessels were frequently found to be injected.

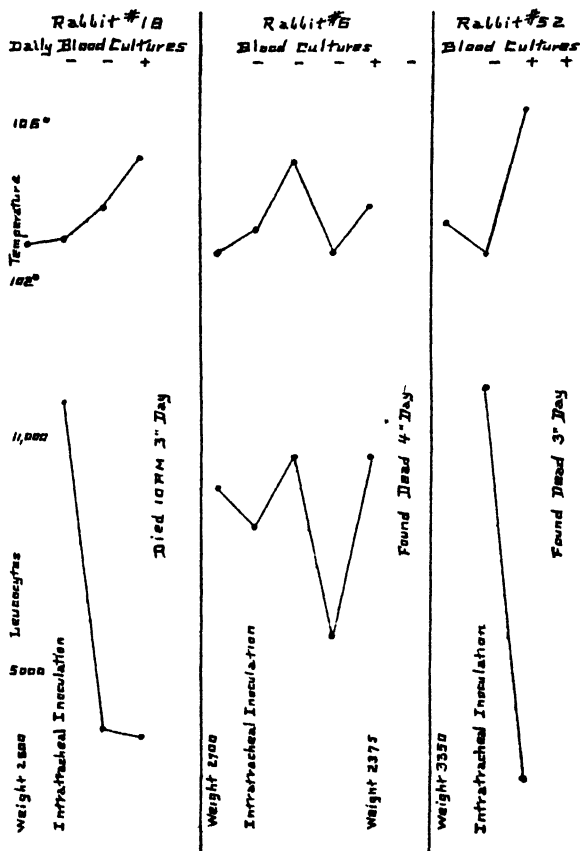


FIG. 2. EFFECT OF PNEUMOCOCCUS INFECTION IN NORMAL RABBITS

*Discussion.* It is of interest that when rabbits were inoculated intravenously with ten small doses of filtrate they developed a sufficient immunity to cause a localization of pneumococcus in the lung after intratracheal inoculation of the test dose. It was realized at the time that with inoculation by the intratracheal



Acute, spontaneous, nephritis was frequently observed and the skeletal muscles showed Zenker's waxy degeneration.

Zenker's waxy degeneration has been observed by Forbus (1926) and Wells (1927) to be present in human beings and mice dying of pneumonia. Since these lesions were present in the animals that did not develop a severe septicemia as well as in those that did it is thought that the lesions were caused by the liberation of a toxic substance in the blood and not by the immediate presence of the pneumococcus in the organs. Haden suggests that the pneumococcus infection causes an accelerated metabolism leading to a toxemia which, in turn, causes the injury to the various organs.

#### RECAPITULATION

1. A saline extract of the pneumococcus prepared by Rosenow's method decreased the phagocytosis of avirulent organism, reduced the opsonic content of serum, only slightly increased the virulence of a borderline culture after it had been transferred several times in the presence of the extract, and failed to influence the virulence of an avirulent pneumococcus.

2. When an extract of the pneumococcus produced by freezing and thawing the organisms was inoculated into white mice it produced purpura. This extract protected mice against an infection with a pneumococcus of low virulence, when the extract had preceded the culture by twenty-four hours. The protection lasted only as long as the purpura was visible. If the extract were given simultaneously with the culture or after a slight infection had developed, fatality resulted. When the extract followed the culture inoculation the purpuric reaction was absent or less severe. The borderline culture, after having been transferred rapidly in a medium which contained some of the extract, was not changed in virulence.

3. Fresh filtrates of the pneumococcus prepared with several different kinds of media were not sufficiently toxic for laboratory animals to cause death but they tended to increase the localization power of avirulent pneumococci when mice were given the two substances simultaneously.

4. The virulence of pneumococci was not significantly effected when they were transferred rapidly in the presence of old culture filtrates which contained a large amount of soluble specific substance, in enriched media and in media which contained substances that lower and raise the electrophoretic potential of bacterial cells.

5. Rabbits inoculated intravenously with ten small doses of pneumococcus filtrate developed sufficient immunity to cause a localization of pneumococci in the lungs.

The authors wish to express their appreciation to Dr. Mercy A. Southwick for the pathological study of the tissues of the rabbits.

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## STUDIES ON RESPIRATORY DISEASES

### XXXV. THE PATHOLOGY OF PNEUMOCOCCUS INFECTIONS IN MICE

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In the preceding paper was reported a study of the influence of some extracts and filtrates of the pneumococcus and of a few chemical reagents on the virulence of pneumococci. It was shown that purpura developed in mice inoculated with the extract prepared by repeatedly freezing and thawing the pneumococci. If the mice received the extract twenty-four hours preceding the inoculation of a pneumococcus culture of borderline virulence, they were protected against the infection. This protection persisted only as long as purpura was observable. It was also reported that when fresh filtrates of virulent pneumococci were inoculated simultaneously with the culture of low virulence a large number of the mice at autopsy showed a fibrinopurulent pleuritis. The most potent filtrates were obtained when the cultures grew in the presence of salts that lower the electrophoretic potential of microorganisms. Complete autopsy records of the mice were kept and the tissues from a representative number were prepared for histological examination. The present paper comprises a study of the pathological lesions observed in these mice.

Among the early reports on the pathological changes produced by the pneumococcus in mice is the report by Sprunt and Leutscher (1912). They studied about eighty mice and found, in those dying several days after injection of living and dead pneumococci, acute degeneration of the walls of some of the large blood vessels and local hemorrhage. They did not find this de-



generation in mice that died from other bacterial diseases. One year previously Leutscher (1911) had reported the finding of large blood clots in one, or in many, pleural regions in mice dying from pneumococcus infections. He stated that the occurrence of the hemorrhage was more frequent with the smallest doses because the duration of life was longer.

In a study of pneumococcus lobar pneumonia in mice, Branch and Stillman (1924) state that the primary lesion is an interstitial inflammation of the alveolar walls and that the infection spreads in the interstitial tissue like a cellulitis.

Julianelle and Reimann (1926) observed hemorrhagic areas in the lungs and voluntary muscles of mice following the injection of the frozen and thawed extract.

In a preliminary report on the pathology of the lungs produced by a filtrate Olson (1925) states that there was intense congestion, interstitial hemorrhage with decreased air content of the alveoli, and interalveolar extravasation but no filling of the alveoli with exudate or blood. There was some cellular reaction along the blood vessels and parenchyma and there was some degeneration of the alveolar epithelium.

The following report of the pathological lesions observed in mice after they had received intraperitoneal inoculations of pneumococci or pneumococcus products, has been divided into five parts.<sup>1</sup>

#### I. THE PATHOLOGICAL LESIONS NOTED IN THE TISSUES OF MICE KILLED AFTER AN INOCULATION OF AN EXTRACT OF PNEUMOCOCCI

In the preceding paper it was reported that, when white mice were inoculated intraperitoneally with an extract prepared by repeatedly freezing and thawing pneumococci, purpura developed on all or a part of the external surface of the mouse that is free from hair. At autopsy it was observed that the injected blood vessels and hemorrhagic areas were not limited to the external surface but that they were present in nearly every organ and tissue of the mouse. The following description of the gross lesions

<sup>1</sup> The detailed tabulations are not included in this report. They are contained in the Doctoral Dissertation of M. J. Pittman, University of Chicago, Libraries.

observed in a mouse which had been killed with chloroform eleven hours after an inoculation of 0.7 cc. of the extract, is in general true for all mice that were killed while purpura was visible. The subcutaneous blood vessels were injected; this was particularly noticeable in the regions of the inguinal and cervical lymph glands. Several hemorrhagic areas were present in the subcutaneous fascia and all the blood vessels of the peritoneum were distended with blood. The liver, spleen and kidney were very dark red in color; and the surfaces of the ovaries and the uterus were a bright red. The blood vessels of the stomach and the omentum were injected. The margins of the lungs were very bright red and extending from the margin towards the hilus were red lines. On the pleura there were many bright red spots, 0.5 to 1 mm. in diameter. The blood vessels of the brain were not injected and the brain appeared to be normal.

The histological study<sup>2</sup> of nine mice, killed after they were inoculated with the extract (table 7 of preceding paper), revealed the same picture of engorged and hemorrhagic blood vessels that had been observed grossly. In the lungs nearly all the blood vessels were distended with blood and there were many areas of hemorrhage and edema. In many, the alveoli were filled with a pink-staining granular exudate and a few leucocytes. These cells were never abundant. There was a slight mononuclear cellular increase in the lungs of all the mice which were killed twenty-four hours after the inoculation, while only one which had received the extract less than twenty-four hours before being killed, showed a mononuclear cellular increase. Compensatory emphysema was present in a third of the lungs.

The other tissues that were examined histologically were the ear, spleen, kidney, adrenals, salivary and cervical lymph glands. The blood vessels of practically all seemed to be distended with blood and some had hemorrhagic areas. The increase in blood was especially noticeable in the sinuses of the spleen and the glomerular tufts of the kidney. The livers from a third of the mice showed an infiltration of polymorphonuclear cells and one had a few necrotic areas.

<sup>2</sup> The tissues were fixed in Zenker's solution embedded in celloidin sectioned and stained with Delafield's hematoxylin and eosin.

## II. PATHOLOGICAL LESIONS OBSERVED IN MICE WHICH DIED AFTER THE INOCULATION OF PNEUMOCOCCI AND A PURPURA-PRODUCING PNEUMOCOCCUS EXTRACT

This division included the study of the gross pathological lesions that were observed in 38 mice and the histological study of the tissues of 9. These mice, which had been inoculated intraperitoneally with the extract and a culture of low virulence, simultaneously or at different time intervals, are from experiments 2, 3, 4, 5, 6, and 8 of the preceding paper and two other experiments not published in detail.

At autopsy, two-thirds of the mice showed external purpura and internally they presented the same picture of engorged and hemorrhagic blood vessels that was observed in the mice killed after they had received the extract alone. In table 1 the frequency of the occurrence of the pathological lesions in these mice may be contrasted. The most marked differences are observed in the hemorrhagic appearance of the lungs and the presence of fibrinopurulent growths. The lungs of 97 per cent of the mice that had received the culture and extract showed hemorrhagic areas grossly while 55 per cent of the mice inoculated with the extract alone showed hemorrhage. The low percentage observed in the latter group is partially due to the fact that external purpura had blanched or was blanching on two of the nine mice before they were killed. Forty-five per cent of the culture extract mice showed a fibrinopurulent pleuritis.

The lungs showed more extensive involvement than the other organs. The picture of the lung was one of marked hemorrhagic areas, edema, consolidation, distention of the capillaries with blood; while some of the larger blood vessels were filled with thrombi. The consolidation which was present in two-thirds of the mice was largely due to edematous fluid, fibrin and a small number of leucocytes.

Again it was observed that there was a polymorphonuclear cellular increase in the liver. Zenker's waxy degeneration was present in the rectus abdominalis muscle of the one mouse in which this organ was examined. This muscle was not examined in the mice which received the extract alone.

### III. PATHOLOGICAL LESIONS OBSERVED IN MICE THAT RECEIVED FILTRATES OF PNEUMOCOCCUS CULTURES

Ten mice were used in this study. Eight were inoculated intraperitoneally with 2 cc. of a fresh filtrate prepared from an eighteen-hour-old broth culture of a highly virulent type I pneumococcus strain. These mice were killed with chloroform, thirty, forty-eight, sixty and ninety hours after the inoculation. At autopsy all showed that the organs were slightly injected with blood. No marked pathological lesions were noted, grossly or microscopically. The liver and kidney showed more hyperemia than any other tissue and the respiratory muscle, rectus abdominalis, of three that were given the filtrate showed slight waxy degeneration. In the other mice this muscle was not examined. The lungs of the filtrate mice showed slightly more consolidation, edema, fibrin, leucocytic exudation, hemorrhage and capillary distention than the control mice, but these lesions were never marked in any of the mice. Compensatory emphysema was present in the lungs of all except one control; and, since this latter condition is frequently observed in normal mice, it is thought that the chronic condition might be partially responsible for the mild pathology observed in the mice.

### IV. PATHOLOGICAL LESIONS NOTED IN MICE INOCULATED WITH PNEUMOCOCCI AND PNEUMOCOCCUS FILTRATES

The tissues were taken from 16 mice used in the experiments of Part III of the preceding paper.

At necropsy the subcutaneous blood vessels and the internal organs showed an increase in blood and some hemorrhage but the hyperemic condition was not as marked as when the mice received the purpura-producing extract.

The most noticeable pathological lesions were the fibrino-purulent growths on the pleurae and to a lesser extent on the peritoneum. Seventy-one per cent of the mice showed a pleuritis, and 33 per cent a peritonitis. The pleuritis was more frequent in the mice which received the pneumococcus filtrate which contained some substance, trivalent salts or serum, that lowers the

electrophoretic potential of microorganisms, than in those which were inoculated with the filtrate of the culture grown in buffered broth or broth containing glucose. Nine of ten mice that died after the inoculation of the former filtrate had a pleuritis.

The pathological lesions of the lungs were more extensive than were those observed in the other groups of mice. The lungs of all showed hemorrhage, consolidation and edema. All except one, showed a moderate amount of leucocytic exudation. The majority showed fibrin and capillary distention while half had thrombi in a number of the larger blood vessels. Interstitial inflammation was not infrequently observed.

Other than the fibrinopurulent growth, the pathological lesions of the peritoneal organs, liver and kidney excepted, were about the same as was reported for those mice that had died after the inoculation of extract and culture. Fifty per cent of the mice had necrotic areas in the liver. The muscle rectus abdominalis which was examined from only two mice, showed waxy degeneration.

#### V. PATHOLOGICAL LESIONS FOUND IN MICE WHICH DIED AFTER THEY HAD BEEN INOCULATED WITH PNEUMOCOCCI OF VARYING DEGREES OF VIRULENCE OF WITH PNEUMOCOCCI GROWN IN THE PRESENCE OF SUBSTANCES THAT LOWER THE P.D. OF MICROORGANISMS

The virulence of the three strains of pneumococci measured in cubic centimeters per MLD for white mice were  $1 \times 10^{-8}$ ,  $1 \times 10^{-3}$  and 1.0 cc. The tissues from only two mice which succumbed to the highly virulent strain were studied microscopically. This number is not sufficient to warrant a generalized statement but it should be mentioned that hyperemia was observed in practically every organ and that the lungs of one showed a picture which closely resembled early lobar pneumonia. A fibrinopurulent pleuritis was not present, nor was it observed at the autopsy of more than 50 other mice which died following an inoculation of this culture.

Of the seven mice which succumbed to the culture of medium virulence (Pn B orig.), more than a third had a fibrinopurulent

pleuritis. The other pathological lesions are very similar to those that were observed in mice that died after they had been inoculated with the culture of lowest virulence (Pn C orig.). From among the mice which died after receiving the latter culture, the tissues of 19 were studied histologically. Again, the same generalized hyperemia was observed as in all mice which received pneumococcus cultures. The lungs showed some edema, consolidation, fibrin, leucocytic exudation, hemorrhage, capillary distention, and some thrombotic blood vessels. Twenty-one per cent of them had a fibrinopurulent pleuritis. To be contrasted with these lesions are those observed in mice which died following the inoculation of the same culture after it had been grown in the presence of substances that lower the P.D. of microorganisms. The substances that were used were cerium and lanthanum nitrate and serum. The latter mice showed more extensive involvement of the lungs and pleuritis was more than twice as prevalent, occurring in 50 per cent of the animals. The lesions of the latter group of mice closely resembled those observed in mice which died from the culture-filtrate inoculations.

#### DISCUSSION AND SUMMARY

This work includes the study of the pathological lesions observed in 138 mice that were killed or died after they had been inoculated with pneumococcus products, pneumococci or the two substances combined. The products of the pneumococci used were filtrates and an extract produced by repeatedly freezing and thawing pneumococci. The pneumococcus cultures, which were selected from strains that varied in virulence, were grown in buffered broth or in broth which contained substances that lower the P.D. of microorganisms.

The gross pathological lesions observed in the majority of the mice are summarized in table 1. On inspection of the table it will be noted that the blood vessels of a very large number of the mice were injected or hemorrhagic and that this condition was most marked in those mice which had been inoculated with broth culture and extract. The extract alone when introduced into mice produced marked hemorrhagic lesions.

TABLE 1  
*Summary of macroscopical findings*

SUBSTANCE INOCULATED	NUMBER OF MICE	SUBCUTANEOUS KNOTS BLOOD VESSELS		INGUINAL LYMPH NODES		CERVICAL LYMPH GLANDS		PERITONEUM BLOOD VESSELS		PLEURAL CAVITY							
		Injected	Hemorrhagic	Enlarged	Injected	Injected	Injected	Injected	Hemorrhagic	Liver	Spleen	Adrenal	Pancreas	Mesenteric blood vessels	Reproductive organs	Fluid	Lung
Extract.....	9	88*		33	33	55				44	88	66	11	66	22	32	55
Ext. + cult.....	38	87	80	8	33	24		43	16	75	20	66	77	28	26	10	97
Filtrate.....	10	40	20	10	10			30		30	70	20	40	40		93	70
Filt. + cult.....	14	79	50	29	29			50	7	35	29	43	64	43	29	36	89
PnC cult.....	19	94	73	21	31	16		47	5	63	5	47	89	47	31	61	21
PnC + salts.....	21	100	63	10	10	5		36		47	52	89		52	16	95	50
PnB cult.....	7	100	66	16	16	16		16		66	33	43	100	66	16	71	43
PnB + salts.....	7	100	83	16	16	16		16		83		16	100	33	16	13	43

\* The figures under pathological headings indicate percentage.

Another finding which is considered significant may also be noted in the table. This is the presence of fibrinopurulent pleuritis among the several groups of mice. The percentages of mice with pleuritis which succumbed to the borderline culture (PnC), the culture plus extract, the culture grown in the presence of substances that lower the P.D. of organisms, and the cultures plus filtrates, are respectively 21, 43, 50 and 71. This suggests that the extract influences to some extent the production of pleuritis, that salts which lower the P.D. are more influential and that filtrates exert the greatest influence. When the mice which received both the culture and the filtrate prepared with a broth that contained substances that lower the P.D., were considered alone, it was noted that 90 per cent of the mice had a fibrinopurulent pleuritis. Since this percentage is so much higher than is observed among the mice which died after receiving large amounts of the culture that had been grown in the presence of these substances, it is thought that there was some other substance in the filtrate influencing the invasion and localization of the pneumococci. Yet this factor is very transient, inasmuch as it had no influence on the virulence of the culture if it preceded or followed the culture inoculation by two hours (table 9 of preceding paper). It may be argued that the salts lowered the virulence of the organisms, thereby giving the pneumococci more time to localize. Yet the same amount of culture when inoculated alone or with broth equivalent to the amount of filtrate, rarely caused death or even an invasion of the blood stream. It should also be mentioned in this connection that the mice frequently died within twenty-four hours (table 8 of preceding paper) and that this interval was less than was sometimes the case when the mice were given high dilutions of the virulent culture. Pleuritis was never observed in mice which died from the effects of the highly virulent culture. It was noted that the culture of medium virulence caused the production of pleuritis in 3 out of 7 mice.

The sections prepared from the lungs of a mouse which had received the highly virulent culture showed a picture that more closely resembled an early stage of lobar pneumonia than was observed in the lungs of other mice. An insufficient number of



mice that died after the inoculation of the virulent culture were examined, however, to draw the conclusion that the most virulent culture produces lobar pneumonia while the ones of lesser virulence produce bronchial pneumonia or localized growths. This conclusion has been drawn by Gaskell (1928), Whittle (1928) and Armstrong and Gaskell (1921). Since no mice showed a complete picture of lobar pneumonia, although the picture at times was lacking only in the massive number of polymorphonuclear cells and heavy fibrin deposits, it is thought that the resistance of the host plays as great a part in the production of lobar pneumonia as the virulence of the culture. The latter theory is supported by the work of Blake and Cecil (1920), Permar (1923), Stillman and Branch (1924), Nakajima (1926) and Pittman and Falk (1930). Stillman and Branch were unable to produce lobar pneumonia in mice unless the mice had a partial immunity to the pneumococcus. They think that interstitial inflammation is the first stage in the development of lobar pneumonia in mice. This lesion was frequently observed in our mice which succumbed to the culture filtrate combination.

A generalized summary of the lesions found in the lungs of mice after they had received pneumococci or pneumococcus products is distention of the capillaries, thrombi in the large blood vessels, edema of the alveolar walls and alveoli filled with an edematous fluid, fibrin and a few leucocytes, and pleuritis. The severity of the lesions found in each group of mice is discussed under the different divisions of the pathological findings. The lungs of that group of mice which were killed after the inoculation of filtrate showed the mildest reaction and the lesions which at no time were marked were never comparable to those reported by Olson. Hemorrhage was most severe in those mice which succumbed to the inoculation of culture and extract; and pleuritis was most frequent in those mice which had been inoculated with culture and filtrate.

It is significant that the most extensive lesions were observed in the lungs when the inoculations were made intraperitoneally. The liver frequently showed an increase in polymorphonuclear cells and the spleen and kidney were engorged with blood. Zenker's waxy degeneration was observed in the respiratory

muscle of all animals examined. This condition is frequently observed in fatal cases of pneumonia and other inflammatory processes (Forbus, 1926 and Wells, 1927).

The experiments have not adequately explained the phenomenon that mice are protected from at least two MLD of a culture of border-line virulence if they have received the purpura-producing extract twenty-four hours before the inoculation. The only difference noted in the tissues of mice that were killed from 24 hours to 5 days after the extract inoculation and in the tissues of those that were killed in less than twenty-four hours after the inoculation, was a slight increase in mononuclear cells in the lungs. Stuppy, Cannon and Falk (1929) and Gay and Clark (1929) have noted a mononuclear cell reaction in the lungs of immune rabbits when they were inoculated with pneumococci. Although our mice had no permanent immunity it might be suggested that this temporary increase in mononuclear cells protected the mice from infection.

As the most extensive hemorrhagic lesions were observed in those mice which died after they received an inoculation of the endocellular substance followed within twenty-four hours by an inoculation of culture, and since the frequency of fibrinopurulent pleuritis was most marked in the mice which died after receiving an inoculation of the culture and the exo-product, it is suggested that both products may function in the pathogenesis of pneumococcus infections. It has been stated (Julianelle and Reimann, 1927) that since purpura is rarely observed in humans or animals with pneumococcus infections this purpura-producing extract is not liberated by the pneumococcus *in vivo*. An argument against this theory was observed in the preceding paper: if the mouse had a pneumococcus infection at the time of the inoculation, or if the extract was given simultaneously with the culture, the external purpuric reaction was less severe or entirely absent.

#### CONCLUSIONS

1. When mice were inoculated with an extract of the pneumococcus produced by repeatedly freezing and thawing the pneumococcus, they developed marked hemorrhagic lesions on all or a part of the external surface that was free from hair, and at autopsy,

after they were killed, hemorrhagic areas could be found in practically every tissue of the mouse.

2. When mice died after they were inoculated with extract followed several hours later with a culture inoculation, they had more marked hemorrhagic lesions than any other group of mice that were studied.

3. Fresh filtrates of virulent pneumococci produced very slight pathological reaction in mice but, when the filtrates were inoculated with a culture of low virulence, 71 per cent of the mice at autopsy showed a fibrinopurulent pleuritis.

4. Pleuritis was twice as frequent in mice which died from pneumococci that had grown in the presence of salts that lower the p.d. as it was in mice which had died from the same strain grown in plain broth.

5. Mice which died or were killed after inoculation of pneumococci or pneumococcus products showed pathological lesions in the lungs, liver, respiratory muscles and possibly in the kidneys.

6. The relation of the extract and filtrates to the pathogenesis of pneumococcus infections is discussed.

The authors wish to express their appreciation to Professor H. G. Wells for assistance with the pathological diagnoses.

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# CERTAIN RELATIONSHIPS OF MARINE BACTERIA TO THE DECOMPOSITION OF FISH<sup>1</sup>

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## OBJECT AND METHOD OF STUDY

In view of the definite relationship of marine organisms to the condition of fishery products (Harrison, 1918, 1926, 1929), it may be helpful to point out some of the fundamental connections between these sea water forms and fish curing problems. Using the plating method with beef-peptone agar, containing 1.5 per cent NaCl, counts of 75 to 200 and over may be obtained during the spring and summer seasons of the year from unpolluted sea water. Samples from the surface and from a depth of 20 meters were taken at ebb tide, in the vicinity of Halifax harbor, a quarter of a mile or more from shore.

The descriptions of the predominating microorganisms and their identification have been attempted. Identification was, on the whole, difficult to accomplish in that certain characteristics exhibited by these forms as a group, differ markedly from the descriptions given for corresponding and related organisms from other sources. In this connection it is important to consider the probable influence of higher concentrations of salt on the growth of marine organisms. In obtaining complete descriptions for purposes of identification, therefore, it is necessary to make certain allowances. For example, as a group, the marine organisms of the genera *Achromobacter* and *Flavobacterium* isolated at this laboratory, do not grow well upon plain potato, though in one or two cases a scant, spreading, translucent growth was detected. If, however, the potato slants are previously immersed in a 3 per cent solution of NaCl, almost all of the organisms develop abundantly with a luxurious spreading growth. Even the micrococci reveal marked cultural differences.

<sup>1</sup> Study from the Stations of the Biological Board of Canada.

Aside from this property several other common differences may be observed, as in types of gelatin liquefaction; the tendency to form slimy or viscous growths; the inability to grow at 37° to 38°C., which does not apply to the majority of the micrococci examined; indifferent behavior toward milk and in fermentation tests; the motility of a number of marine cocci.

#### TYPES OF MARINE BACTERIA AND THEIR PROTEOLYTIC POWER

The following list includes those species which the bacteria investigated resemble most closely. In two cases a name has been suggested where the occasion seemed to justify.

*Micrococcus candidus* Cohn  
*Micrococcus citreus* Migula  
*Micrococcus conglomeratus* Migula  
*Micrococcus conglomeratus* var.<sup>2</sup>  
*Micrococcus varians* Migula

*Flavobacterium turcosum* (Zimmerman) Bergey et al.  
*Flavobacterium sulfureum* Bergey  
*Flavobacterium aurescens* (Ravenel) Bergey et al.  
*Flavobacterium annulatum* (Wright) Bergey et al.  
*Flavobacterium brevis* (Frankland) Bergey et al.

*Flavobacterium pruneaeum* Sanborn N. Sp.  
*Flavobacterium gelatinum* Sanborn N. Sp.

*Achromobacter raveneli* (Chester) Bergey et al.  
*Achromobacter litoralis* (Russell) Bergey et al.  
*Achromobacter formosum* (Ravenel) Bergey et al.  
*Achromobacter liquidum* (Frankland) Bergey et al.  
*Achromobacter geniculatum* (Wright) Bergey et al.

	<i>Flavobacterium pruneaeum</i> Sanborn N. Sp.	<i>Flavobacterium gelatinum</i> Sanborn N. Sp.
Rods:	2.0-3.0 x 0.5 $\mu$ . Occur singly. Rounded ends. Spherical forms observed. Gram-negative. Flagella polar (monotrichous). (Nachtblau; Casares-Gil's method.)	3.3-4.9 x 0.5-0.8 $\mu$ . Occur singly. Rounded ends. Beaded and granular forms. Gram-negative. Peritrichous. Semi-gelatinous envelope obscures flagella which number 3 to 5 or more (Casares-Gil's method).

<sup>2</sup> This strain exhibits minor differences and does not reduce nitrates.

<b>Agar colonies:</b>	Growth rapid, circular smooth, raised, edge entire translucent, yellowish-orange, viscid.	Growth slow, circular, smooth, cup-shaped liquefaction; center yellowish-orange, margin translucent.
<b>Agar stroke:</b>	Echinulate, raised, glistening, contoured, orange to pink, margin translucent and lighter in color, viscid.	Spreading, glistening, fluorescent, smooth, butyrous, yellow to orange; agar dissolved.
<b>Gelatin colonies:</b>	Growth rapid, circular, flat, erose, orange to brown, saucer-shaped liquefaction.	Growth slow, circular, crateriform, saucer-shaped liquefaction, internal structure finely to coarsely granular.
<b>Gelatin stab:</b>	Surface growth orange to pink; slow liquefaction; crateriform in 13 days, becoming stratiform.	Liquefaction crateriform in 5 days; stratiform in 7 days. Medium browned.
<b>Broth:</b>	Clouding slight; sediment moderate and viscid on agitation. Viscid pellicle forms.	No pellicle; clouding slight, moderate amount of sediment.
<b>Potato (plain):</b>	No growth.	Scant growth.
<b>Potato (saline):</b>	Abundant, spreading, smooth, glistening, light terra cotta to apricot red. Medium darkened.	Scant growth.
<b>Litmus milk:</b>	Slightly acid in 10 days. Reduction.	Unchanged.
<b>Purple milk:</b>	Slightly acid in 10 days (very slow peptonization).	Unchanged.
<b>Glucose:</b>	—	—
<b>Lactose:</b>	—	—
<b>Sucrose:</b>	—	—
<b>Nitrate broth:</b>	No reduction. Viscosity.	Reduction.

Indol:	Not formed.	Not formed.
Lead acetate agar:	Abundant, lobate, pulvinate, smooth, slightly viscid, buff to light brown becoming pinkish brown. Medium browned.	Moderate, echinulate, viscid, smooth, orange-yellow. Medium unchanged in color.
Temperature:		
38°C.:	No growth.	No growth.
12°C.:	Good growth.	Scant growth.
20°C:	Good growth.	Good growth.

For the purpose of comparison it will prove helpful to list the marine organisms isolated by Harrison (1929) from living and dead halibut.

*Micrococcus varians* Migula  
*Micrococcus flavus* Lehmann and Neumann  
*Micrococcus aurantiacus* (Schröter) Cohn  
*Sarcina lutea* Schröter  
*Flavobacterium fucatum* Harrison  
*Flavobacterium turcosum* (Zimmerman) Bergey et al.  
*Flavobacterium dormitor* (Wright) Bergey et al.  
*Flavobacterium diffusum* (Frankland) Bergey et al.  
*Micrococcus citreus* Migula or  
*Staphylococcus citreus* Bergey et al.  
*Micrococcus candidus* Cohn  
*Rhodococcus agilis* (Ali Cohen) Holland  
*Flavobacterium maris* Harrison  
*Flavobacterium balustinum* Harrison  
*Flavobacterium marinum* Harrison  
*Achromobacter pellucidum* Harrison  
*Aerobacter cloacae* Jordan

Harrison has also reported the appearance of a number of organisms in fish muscle which are probably marine forms (Harrison, 1926). Among these there are several mentioned above, e.g., *A. formosum*, *A. liquidum*, and *A. geniculatum*. He associates the members of the genus *Achromobacter* with more or less definite decomposition changes taking place in fresh fish.

In the fishery industry the view is often taken that there are probably two types of decomposition in which microorgan-

isms may engage. One is a proteolytic action, taking place at relatively low temperatures and somewhat related to autolysis. The result is a softening and structural breakdown of tissues observed in dead and at times in living fish. It is possible that the bacteria of the *Flavobacterium*, *Achromobacter*, *Pseudomonas*, and *Micrococcus* groups may conduct this type of decomposition. Then there are the microbial decompositions described as putrefactive, taking place at ordinary temperatures and involving a number of genera: *Escherichia*, *Proteus*, *Salmonella*, *Eberthella*, *Micrococcus*, and *Pseudomonas*.

One method of observing the proteolytic effect is by the development of clear zones on a fresh fish agar plate. Cooled, melted B.P. agar is poured over a mascerated suspension of fresh fish in a petri plate. The latter is prepared under aseptic conditions and can be preserved for some time, frozen and thawed alternately during the period of use. It was found helpful to employ a 1:1000 concentration of brilliant green in the suspension.<sup>3</sup> The organism is streaked upon the solid medium and within three or four days a definite clear zone appears about the growth, while the rest of the medium retains the turbidity imparted to it by the fish suspension. The following results were obtained with the marine forms. Two additional organisms isolated from the muscle of fresh cod, are included.

Zone development in 4 days  
mm. in width

<i>Micrococcus conglomeratus</i> Migula.....	10-13
<i>Micrococcus conglomeratus</i> var.....	7-10
<i>Flavobacterium aurescens</i> (Ravenel) .....	8-9
<i>Micrococcus subcitreus</i> Migula (from cod) .....	6-7
<i>Micrococcus citreus</i> Migula .....	5-8
<i>Flavobacterium sulfureum</i> Bergey. . . . .	4-6
<i>Pseudomonas fluorescens</i> Migula (from cod).....	3-5
<i>Flavobacterium annulatum</i> (Wright).....	3-5
<i>Flavobacterium pruneaeum</i> (Sanborn) N. Sp.....	2

The other sea water organisms exerted little effect. In contrast to these results, certain bacteria associated with definitely putrefactive processes did not produce this reaction. These were iso-

<sup>3</sup> One cubic centimeter of suspension used with 10 cc. melted agar.



lated from fish undergoing decomposition and respond closely to the descriptions of the following organisms:

*Eberthella talavensis* Castellani and Chalmers (from cod showing green pigmentation)

*Salmonella morgani* Castellani and Chalmers (from spoiled haddock; completely reduces methylene blue in fresh fish media in 16 hours)

*Escherichia formica* Omelianski (from spoiled haddock)

Washings from the visceral cavity of split fish revealed an abundant contamination with organisms belonging to the intestinal genera which may have originated in the sea or from sources of pollution more or less remote. These groups are particularly abundant in the slime and blood distributed about the visceral cavity. It is probable that many of these organisms are from the alimentary canal of the fish and exert a decided influence upon the keeping quality of the product.

#### MICROÖRGANISMS ASSOCIATED WITH THE DECOMPOSITION PROCESSES OF FRESH SMOKED HADDOCK

Hess (1929) states that non-spore-forming bacteria may be killed by smoke (produced by the burning of sawdust) under experimental conditions, in from one to three hours depending upon the density of the smoke. The smoking of haddock for five hours exerts a definite bactericidal effect. In one experiment carried on under controlled conditions it was found that practically the only organism capable of withstanding the smoking for this period of time was *Achromobacter raveneli*, previously referred to as a marine form. On exposure to the air, smoked haddock almost invariably encourages the rapid development of *Penicillium*. In several cases an alcohol-forming torula was isolated. The torula grows upon the surface of the fish in dry, raised, white areas. It develops well in maltose, sucrose, xylose, mannitol, galactose, and levulose broths, and upon B.P. agar, potato, and fresh fish. There was no liquefaction of gelatin. The cells are spherical, 2.5 to 5.0 microns in diameter (size of majority 3.3 microns). Guilliermond (1920) refers to torulae found in sea water and their presence in pickle brine. The latter observation has been confirmed at this laboratory.

Among the bacteria isolated from spoiled smoked haddock, the following forms were identified:

*Achromobacter geniculatum* From sea water; associated with torulae.

*Achromobacter litoralis* From sea water; apparently not putrefactive.

*Micrococcus varians* From sea water; associated with other cocci.

*Micrococcus perflavus* Orange pigmented growth abundant on fillet.

*Rhodococcus agilis* Grows more slowly. Fish reveals pink pigment more or less mixed with the orange. Isolated from skin of halibut (Harrison 1929).

#### MICROÖRGANISMS PRESENT IN SPOILED ICE FILLET

The bacteriological examination of spoiled ice fillets (cod) stored at  $-5^{\circ}\text{C}.$ , reveals the presence of a number of psychrophilic organisms. Contamination may occur from sea water and body slime; alimentary tract of fish; ice; handling; paper wrapper; utensils.

The fillets were about ten months' old at the time of the examination and showed definite signs of decomposition. Black spots were present, suggesting the activities of filamentous fungi; yellow areas and small red spots pointed to active bacterial development. Growth also extended to the paper wrapper. The following organisms may be held responsible for the major portion of this spoilage at low temperatures.

*Proteus vulgaris* Hauser. Highly putrefactive; capable of withstanding  $-18^{\circ}$  to  $-23^{\circ}\text{C}.$ , for months. Grows with great rapidity if transferred to ordinary temperatures during this time. Probably from alimentary tract.

*Chaetostylum Fresenii*. Grows rapidly over the surface of fresh fish. Gives distinct odor. Capable of withstanding  $-18^{\circ}$  to  $-23^{\circ}\text{C}.$ , for months. Possibly from wax paper wrapper.

*Eberthella bienstockii* Schröter. Putrefactive; capable of withstanding  $-18^{\circ}$  to  $-23^{\circ}\text{C}.$  for months. Probably from alimentary tract.

*Micrococcus subcitreus* Migula. Grows rapidly over surface of fresh fish with the production of a bright yellow pigment. Gives distinct odor. Capable of withstanding  $-18^{\circ}$  to  $-23^{\circ}\text{C}.$ , for months. Possibly marine.

*Micrococcus candidus* Cohn. Capable of withstanding  $-18^{\circ}$  to  $-23^{\circ}\text{C}.$ , for months. Marine.

#### Associated species:

*Micrococcus citreus* Migula. Marine.

*Achromobacter refractum* (Wright) Probably marine and non-putrefactive.

## SUMMARY AND CONCLUSIONS

Marine bacteria are consistently present in and on fresh fish and in smoked and frozen fish. They are active at relatively low temperatures and many of them engage in proteolytic processes in fish muscle.

Organisms belonging to the intestinal genera are invariably present in the visceral cavity of split fish and probably come from the alimentary tract. Some definitely putrefactive forms are included in this group which are active in the decompositions of fresh and frozen fish. The removal of contaminating material from the visceral cavity by washing is a wise precaution. Improved and more careful methods of handling will probably result in better keeping quality.

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# THE FORMULA FOR THE FERMENTING CAPACITY OF A SINGLE CELL

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In his criticism of the paper of Bayne-Jones and Rhees (1929), Wetzel (1929) claims that the rate of heat production of a single bacterium in these experiments cannot be represented by the Buchanan formula. Since there is no essential difference between heat production and the formation of any other fermentation products, the Buchanan formula could not be applied anywhere if Wetzel's criticisms were justified. The author believes, however, that Wetzel's deductions have missed the essential point. To understand this, we must go back to the historical development of the formula.

If multiplying cells cause a fermentation, the rate of fermentation can be stated correctly only by determining the amount of product formed per cell and per hour. This can be estimated by taking the average between the initial count of bacteria  $a$  and the final count  $b$ , and dividing the total amount of products  $S$  by this average and by the time  $t$ . Thus, the fermenting capacity per cell and hour would be

$$x = \frac{S}{t \cdot \frac{1}{2} (a + b)} = \frac{2 S}{t (a + b)}$$

Since the simple average does not actually present the average number of fermenting cells in a rapidly multiplying culture, Rahn (1912) developed the following formula

$$x = \frac{S (\log b - \log a)}{t (b - a) 0.301}$$

This formula was developed and used for short time intervals only;  $t$  did not mean the entire time of the experiment, but the

duration of one time interval between two counts. Consequently,  $a$  was not the number of cells at the start, but the number at the beginning of the time interval under consideration.  $S$  was the *increase* of products during this time interval. For short time intervals, Rahn assumed the output per cell to be constant.

This formula was improved upon by Buchanan and Fulmer (1918) who, by introducing calculus, came to the expression

$$x = \frac{S (\log b - \log a)}{t (b - a) \cdot 0.434}$$

Whether these authors meant this formula to be applied for short intervals only, is not stated, and they did not apply the formula themselves.

Bayne-Jones and Rhees (1929) did not use this formula for each interval separately, but computed the heat produced per cell and per hour by always using the values at the time 0 as initial values. Since the output per cell and hour certainly cannot be considered constant during the entire growth period, Wetzel's objection to this use of the formula is justified. It might be better, therefore, to formulate this expression a little differently so as to suggest its application for each time interval separately by writing it

$$x = \frac{\Delta S (\log b_2 - \log b_1)}{\Delta t (b_2 - b_1) 0.434}$$

where  $\Delta S$  and  $\Delta t$  mean increases during a short time interval, and  $b_2$  and  $b_1$  the corresponding bacterial counts. If this formula is applied to each interval separately, the data in table 1 are obtained from Bayne-Jones and Rhees' values.

Wetzel equates the formulas from which the columns 5 and 6 are computed, and obtains the result that a variable equals a constant, which means, that the two formulas can not be compared. This result was to be expected because the one formula is known to be more accurate than the other, and consequently both cannot give the same result.

The author (1929) has recently called attention to the fact that the formula of Buchanan or Rahn applies only as long as the rate

TABLE 1  
*Calories produced per cell and per hour*

TIME	CALORIES PRODUCED IN THE MEDIUM		CELLS PER FLASK	CALORIES PER CELL COMPUTED			
	Total	Increase per hour		For the entire time by the formula		For each interval by the formula	
	$S$	$\Delta S$		$\frac{S}{t}$	$\frac{2.303S \left( \log \frac{b}{a} \right)}{t(b-a)}$	$\frac{2\Delta S}{\Delta t(b_2 + b_1)}$	$\frac{2.303 \cdot \Delta S \left( \log \frac{b_2}{b_1} \right)}{\Delta t(b_2 - b_1)}$
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
<i>hours</i>							
0	0	0	$360 \times 10^6$	—	—	—	—
1	1 485	1 485	420	$3.54 \times 10^{-9}$	$3.72 \times 10^{-9}$	$3.81 \times 10^{-9}$	$3.72 \times 10^{-9}$
2	17 68	16 20	2 160	8 18	8 77	12 56	15 25
3	50 29	32 61	9 600	5 24	5 95	5 55	6 54
4	76 65	26 36	33 000	2 28	2 65	1 22	1 36
5	92 73	16 08	96 000	0 97	1 08	0 25	0 27
6	108 60	15 87	129 000	0 84	0 83	0 14	0 14

of multiplication is increasing or constant. If the growth curve is drawn by plotting the number of bacteria (not the logarithms) against time, these formulas will be correct until the inflexion point is reached; beyond that, the arithmetical average is more accurate than the formulas.

Wetzel further develops a new formula for the fermenting capacity. The basis of this formula

$$x = \frac{1}{b} \frac{dS}{dt} = \frac{\alpha}{B} 2^{-\frac{t}{\theta}}$$

is the observation that, in the data of Bayne-Jones and Rhees, the total calories plotted against time give a curve which is slowly rising for the first hour, and then becomes almost linear for a period of three or four hours or even longer. This straight-line relation is expressed in an equation which is combined with other equations to develop the final formula. Wetzel does not try to account for this linear curve which means that the total heat output is proportional to the age of the culture. The curves show plainly that this holds true only for a short time interval of three to four hours when the number of cells is between 20,000,000 and 300,000,000 per cubic centimeter. Before this number is reached, the heat formation is faster, and afterwards, it is slower. The linear agreement is therefore nothing but a somewhat extended point of inflexion of the typical sigmoid curve of fermentation products. Such extended points of inflexion are frequently found (see, e.g., Rahn, 1910) but represent only a small part of the entire fermentation period. Such a basis is rather weak because no biological reason can be given why the heat output should be proportional to time, while the formulas of Rahn and Buchanan are based on our experience of the multiplication of bacteria, and make only the assumption that during the time interval under consideration, the output per cell is constant. This assumption can be met experimentally by choosing short time intervals. Wetzel's criticism that "the rate of heat production of a single bacterium cannot be represented in these experiments by the Buchanan formula" is correct only if the Buchanan formula is

applied for long periods during which a considerable change of the rate of fermentation or heat production takes place.

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# THE INFLUENCE OF FIXED NITROGEN ON AZOTOBACTER

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The nitrogen-fixing organism, *Azotobacter*, has been little studied with reference to its normal physiological behavior in fixed nitrogen, and especially in comparison with that in free nitrogen. A knowledge of the physiology of *Azotobacter* growing in readily available fixed nitrogen is highly essential to controlling the interpretation of the physiological behavior of *Azotobacter* when fixing nitrogen, particularly in connection with ascertaining the nature of the chemical mechanism of nitrogen fixation. In accordance with this logical principle, Burk (1930) has already shown, for example, that the unique oxygen pressure functions of *Azotobacter* with respect to respiration, growth, nitrogen fixation, efficiency of growth, efficiency of nitrogen fixation, pH, and humic acid are qualitatively identical whether the nitrogen supply is either free or fixed, and hence that these functions do not concern directly the chemical mechanism of fixation.

Bonazzi (1924), Zoond (1926), Kostyschew, Ryskaltshuk and Schwezowa (1926), and others have shown that *Azotobacter* is able to utilize nitrates, ammonium salts, amino acids, and peptones, etc., in preference to free nitrogen, and that fixation is, indeed, not an essential function. In establishing the quantitative aspects of these relations, however, previous investigators have been handicapped by the lack of a suitable manometric technique, and have employed the older chemical methods involving nitrogen and sugar analyses. Their experiments usually required several days or weeks, rather than a few hours, so that it was impossible to maintain constant, reproducible

conditions, and the ambiguous effects of old or heavy growths inevitably obtained, i.e., marked decreases in growth rate referable to mere numbers only, relative lack of nutrients circumstantially consumed, or complicated mixtures of various stages of life cycles.

It is the chief task of the present paper to analyze the normal metabolic and growth functions of *Azotobacter* maintained in fixed nitrogen, apart from those functions with respect to oxygen pressure which have already been considered elsewhere (Burk, 1930). The term "fixed nitrogen" will ordinarily designate "rapidly available fixed nitrogen," and will not refer to completely unavailable or slowly available nitrogen compounds.

#### I. METHODS

A complete description of the technique employed will be found in a previous paper (Burk, 1930). In brief, oxygen consumption has been measured according to the quantitative, physico-chemical, manometric micro-methods for studying cell metabolism, well worked out and described by Otto Warburg (1926). Figures 1 and 2 show the particular type of manometer and vessel used. The vessels are filled at atmospheric pressure with various gases by passing the latter for two or three minutes down through the manometer stopcocks and out through the glass-ground necks of the side-cups, and then closing the two latter openings simultaneously. Mixtures of gases are made by means of a calibrated, multiple, all-glass flowmeter. The amounts of oxygen consumed are ordinarily too small to alter the composition of the gas appreciably. The total volume of a vessel (fig. 2) is about 16 cc., 2.00 cc. of which is occupied by the culture medium, and 0.30 cc. by the 2 N NaOH in the alkali container. Before occupying the manometer vessels, the experimental organisms are first grown in aerated gas wash bottles at 28°C. for twenty-four to forty-eight hours in a medium of the following composition: 0.8 gram  $K_2HPO_4$ , 0.2 gram  $KH_2PO_4$  (pH 7.3); 0.2 gram  $MgSO_4$ ; 0.2 gram NaCl; 0.1 gram  $CaSO_4$ ; 0.01 gram  $Fe_2(SO_4)_3$ ; 10 grams glucose; 1000 grams water. Usually such cultures are diluted somewhat with fresh medium immediately before use. The

manometers are shaken at the rate of 120 cycles per minute, with an amplitude of 3 cm.; this provides a maximum respiration rate and adequate equilibrium conditions between gas and liquid phases. The manometer readings, multiplied by a constant characteristic for each vessel, give directly the number of cubic millimeters of oxygen respired; the readings are accurate to about  $\pm 0.5$  c.mm. The growth of bacteria is measured directly by counting the number of cells before and after an experiment by means of either Hawksley-Thoma or Petroff-Hausser haemocytometers, the depths of which are 20, instead of the usual 100, microns. Before counting, the pH of a culture is brought to 5, to prevent all movement and growth. Two species of *Azotobacter* have been used, *A. chroococcum* Strain SM 1, obtained directly from the Rothamsted Experimental Station (England), and *A. vinelandii*, obtained originally from the New Jersey Agricultural Experiment Station.

## II. THE RATIO OF GROWTH TO RESPIRATION RATE INCREASE

Whenever an inoculum is seeded into a series of vessels kept filled with gas of constant oxygen pressure, and variously treated with humic acid, ammonia, or any other reagent so that the subsequent rates of growth differ, temporal increases in the number of organisms accompany temporal increases in the rates of oxygen consumption (and likewise temporal increases of total oxygen consumption) in the same respective order. This important relation is based upon over one hundred and fifty observations. It means that by measuring the rates of respiration for merely a few hours one can determine qualitatively, and semi-quantitatively, the amount of growth and (in cases where the nitrogen supply is  $N_2$ ) of nitrogen fixation.

The constant of proportionality between growth and respiration rate increase is not exactly the same for every culture of the series, however, but increases as the slope of the respiration rate time curve increases. This is true because, as will be shown, the efficiency of growth increases with the rate of growth. The relative differences between the deduced growths are therefore somewhat greater than the observed increases in respiration rate

would make them appear. In addition to the practical application mentioned above in connection with approximating growths from respiration rates, it is important in interpreting the mecha-

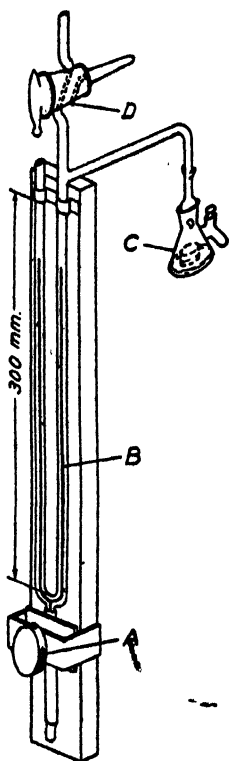


FIG. 1

FIG. 1. WARBURG-BARCROFT MANOMETER

A, screw pinchcock; B, manometer fluid in graduated capillary tube; C, respiration vessel; D, two-way stopcock. All glassware Pyrex.

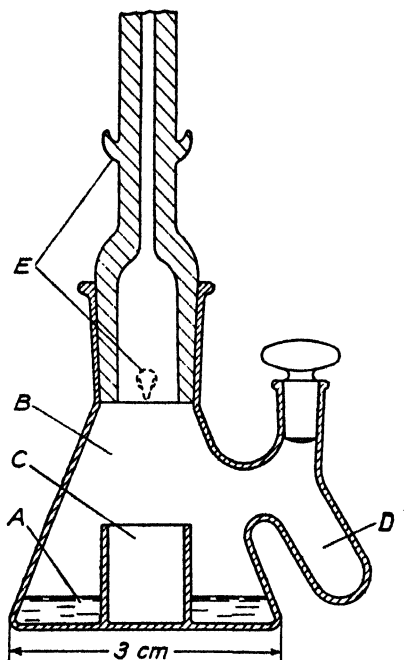


FIG. 2

FIG. 2. RESPIRATION VESSEL

A, culture medium (liquid phase); B, gaseous phase; C, container for  $\text{CO}_2$ -absorbing alkali; D, slide-cup; E, glass prongs for wire springs holding vessel on to manometer.

nism of fixation to determine how the constant of proportionality varies with different factors in order to ascertain whether the constants increase comparably in free and fixed nitrogen.

The constant of proportionality will be defined as

$$k = \frac{\text{final count} - \text{initial count}}{\text{initial count}} \div \frac{\text{final respiration rate} - \text{initial respiration rate}}{\text{initial respiration rate}}$$

$$= \frac{\text{growth}}{\text{initial count}} : \frac{\text{respiration rate increase}}{\text{initial respiration rate}}$$

This constant is independent both of the units employed in measuring the count and oxygen consumption, and the amount of initial inoculum, and it approaches zero as a lower limit.

TABLE 1

*k* as a function of nitrogen pressure, in the presence and absence of humic acid

A. Per cent of nitrogen gas.....	0	3	10	25	45	78
B. Final respiration rate, no humic acid, 0-13 hours.....	23.8	23.9	32.5	66.3	98.8	100
C. Final respiration rate, humic acid, 0-13 hours.....	23.1	40.0	75.6	199	318	339
<i>k</i> { No humic acid, 0-13 hours.....	0	0	0.302*	0.288	0.467	0.696
Humic acid, 0-13 hours.....	0	0.347	0.596	0.635	0.662	0.850

Initial respiration rate, no humic acid, 23 c.mm. per 2 cc. per hour.

Initial respiration rate, humic acid, 25 c.mm. per 2 cc.

Initial count, 24 million per 2 cc.

1½-day old *A. chroococcum* Strain SM 1, diluted 3 times.

20 per cent oxygen, various percentages of nitrogen, hydrogen to make up to 1 atmosphere.

0.25 mgm. humic acid per 2 cc., when present.

\* Slightly high.

These advantages are not possessed by the following ratios, which might otherwise have been used: (final count/initial count)/(final respiration rate/initial respiration rate); (final count - initial count)/(final respiration rate - initial respiration rate). The first of the latter two approaches 1 as a lower limit, which often results in a minimum function that is physiologically, even if not mathematically, misleading. The second is not a relative ratio, but depends upon the units employed and amounts of initial inoculum. While fairly close qualitative agreement is

TABLE 2  
*k* as a function of concentration of humic acid

A. Milligram humic acid per 2 cc.	0	0.012	0.037	0.111	0.333	1.00
B. Final respiration rate.	0-11 hours	84.0	117	135	156	168
	0-14 hours	87.0	207	251	272	304
<i>k</i> .....	0-11 hours	0.435	0.515	0.625	0.745	0.785
	0-14 hours	0.780	0.900	1.07	1.25	1.25

Initial respiration rate, 15 c.mm. per 2 cc. per hour.

Initial count, 8 million per 2 cc.

1-day old *A. chroococcum* Strain SM 1, diluted 2 times.

21 per cent oxygen in nitrogen.

TABLE 3  
*k* as a function of fixed nitrogen

A. Milligram NH <sub>3</sub> -N per 100 cc.	0	0.10	0.30	0.50	5.00
B. Final respiration rate.....	0-9 hours	20.4	44.1	46.6	48.7
	0-12 hours	21.1	41.2	55.0	67.1
<i>k</i> .....	0-9 hours	0	0.314	1.73	2.81
	0-12 hours	0	1.03	2.96	4.18

Initial respiration rate, 23 ± 3 c.mm. per 2 cc. per hour.

Initial count, 22 million per 2 cc.

3-day old *A. chroococcum* Strain SM 1, diluted 3 times.

21 per cent oxygen in hydrogen.

TABLE 4  
*k* as a function of free and fixed nitrogen, in the presence and absence of humic acid

A. Source of nitrogen.....	N <sub>2</sub>	N <sub>2</sub> , humic acid	NH <sub>3</sub> -N	NH <sub>3</sub> -N, humic acid
B. Final respiration rate.	0-10 hours	63.8	82.3	85.0
	0-13 hours	105	160	188
<i>k</i> .....	0-10 hours	0.658	1.70	2.81
	0-13 hours	0.643	1.53	4.94

Initial respiration rate, 8 c.mm. per 2 cc. per hour.

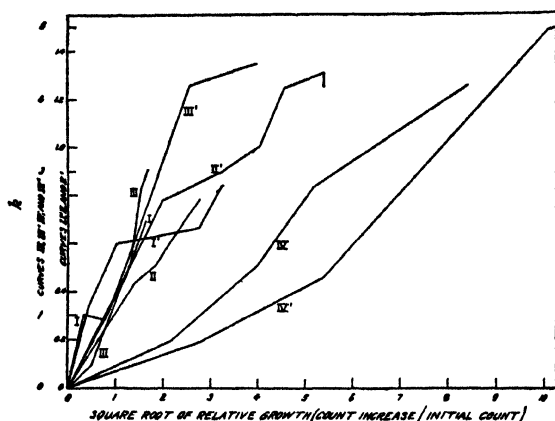
Initial count, 5 million per 2 cc.

1-day old *A. chroococcum* Strain SM 1, undiluted.

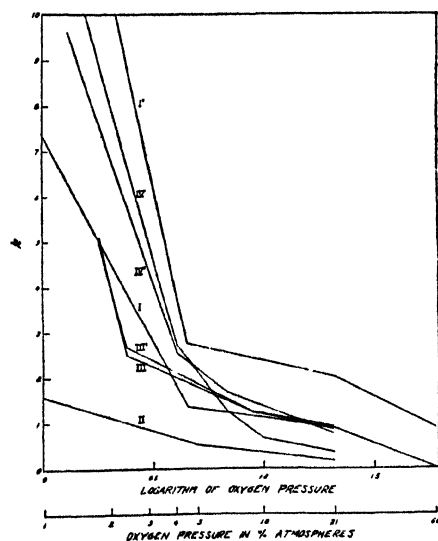
21 per cent oxygen in nitrogen.

0.25 mgm. of humic acid per 2 cc., when present.

0.1 mgm. of NH<sub>3</sub>-N per 2 cc., when present.

FIG. 3. INFLUENCE OF RATE OF GROWTH ON  $k$ 

Curves I and I', II and II', III and III', and IV and IV' based on values of  $k$  given in, respectively, tables 1 (no humic acid and humic acid), 2 (0-11 and 0-14 hours), 3 (0-9 and 0-13 hours), and 4 (0-10 and 0-13 hours).

FIG. 4.  $k$  AS A FUNCTION OF OXYGEN PRESSURE

Curves I and I', II, III and III' and IV and IV' based on values of  $k$  given in, respectively, tables 5 (0-8 and 0-13 hours), 6, 7 (count and nitrogen content), and 8 (count and nitrogen content).



obtained with all three constants, the first is specific and decidedly more fundamental.

It will be seen in tables 1 to 4 that  $k$  always increases with the final respiration rate. In table 1 the final respiration rate has been varied by changing the pressure of nitrogen, and by the presence of humic acid; in table 3, by changing the concentration of fixed nitrogen in the absence of free nitrogen; in table 2, by changing the concentration of humic acid; and in table 4, by the presence of humic acid and fixed nitrogen. Figure 3 (plotted as a square root function to reduce abscissal spread) shows that  $k$  also always increases with growth rate, as well as with final respiration rate; this is, indeed, the more fundamental relation, since final respiration rate is itself a function of growth, rather than *vice versa*.  $k$  does not depend solely upon rate of growth, however, since the various curves are quantitatively different. Increases of  $k$  in free and fixed nitrogen are qualitatively comparable, it should be observed.

For experiments lasting  $10 \pm 2$  hours,  $k$  varies approximately according to the following conditions, each being at optimum.

$N_2-N$ .....	$\frac{1}{2}$ to 1
$N_2-N$ , humic acid.....	1 to 2
$NH_4-N$ .....	2 to 4
$NH_4-N$ , humic acid.....	4 to 6

These numerical values reflect how these conditions influence growth even more than final respiration rate, since the former is affected much more by the various conditions, than the latter. In general, humic acid doubles the rate of growth in either free or fixed nitrogen, while fixed nitrogen doubles the rate of growth as compared with that in free nitrogen.

Tables 5 to 8 and figure 4 show that when the oxygen pressure is varied,  $k$  no longer invariably increases regularly with respiration rate increase (nor, as may be merely stated, with growth), but increases rapidly as the oxygen pressure is decreased. Figure 4 has been plotted semi-logarithmically in order to condense the abscissal spread of the curves. Comparison of Curves I and I' shows that  $k$  increases with duration and time at all oxygen pressures. Comparison of curves II and I shows that  $k$  decreases

with the age of the initial inoculum. Comparison of Curves III and III', or IV and IV', shows that in general little difference is noted, in the case of free nitrogen, whether  $k$  is based upon nitrogen or count determinations, at least in young, highly diluted cultures. Comparison of curves III, III', and I shows that for inocula of the same age, and experiments of the same length,  $k$

TABLE 5  
*k* as a function of oxygen pressure (in fixed nitrogen)

A. Per cent of oxygen in nitrogen.....	60	21	4.5	1.0	0.1
B. Final respiration rate.....	0- 8 hours	15.0	78.9	42.6	14.0
	0-13 hours	40.0	216	113	15.0
C. Initial respiration rate (c.mm. per 2 cc. per hr.).....		15.0	17.4	12.8	11.6
<i>k</i> .....	0- 8 hours	0	0.896	1.36	7.33
	0-13 hours	1.40	2.01	2.75	18.1

Initial count, 12 million per 2 cc.

2-day old *A. chroococcum* Strain SM 1, diluted 3 times.

0.1 mgm.  $\text{NH}_3$ -N per 2 cc.

TABLE 6  
*k* as a function of oxygen pressure (in fixed nitrogen)

A. Per cent of oxygen in nitrogen...	21	5	1	0.25	0.25 (No $\text{NH}_3$ -N)
B. Final respiration rate, 0-8 hours..	150	89	34	7.3	8.2
C. Initial respiration rate, 0-8 hours.	18.2	17.1	13.9	7.3	8.2
<i>k</i> , 0-8 hours.....	0.166	0.523	1.59	$\infty$	$\infty$

Initial count, 50 million per 2 cc.

6-day old *A. chroococcum* Strain SM 1, diluted 7 times.

0.1 mgm.  $\text{NH}_3$ -N per 2 cc., except in case of last column, where none.

varies quite similarly in both free and fixed nitrogen. Indeed, a smooth curve may be drawn through all points of these three curves (see Burk, 1930, fig. 2, curve B, also).

Since  $k$  is defined as a direct fractional function of two other functions which increase logarithmically with time, it is inevitable that  $k$  should change with time, and, therefore, duration, when

the constants of logarithmic (or likewise geometric) increase are different for each function of the fraction, as is the case with *Azotobacter*. In tables 1 to 5 there is no indication, even in the experiments of longest duration, of  $k$  having reached a

TABLE 7  
*k* as a function of oxygen pressure (in free nitrogen)  
Based upon both count and nitrogen determinations

A. Per cent oxygen in nitrogen.....	21	9	2 4	1.8
B. Final respiration rate, 0-8 hours.....	44	106	33	12
C. Initial respiration rate, 0-8 hours .....	18	12	11	6
$k$ (Based on count), 0-8 hours.....	0.865	1.23	2.50	5.12
$k'$ (Based on nitrogen content), 0-8 hours.....	0.924	1.23	2.67	5.00

Initial count, 8 million per 2 cc.

Initial nitrogen, 0.6 mgm. per 2 cc. (as bacterial nitrogen).

2-day old *A. chroococcum* Strain SM 1.

$k' = (\text{nitrogen fixed/initial nitrogen content})/(\text{respiration rate increase/initial respiration rate})$ .

TABLE 8  
*k* as a function of oxygen pressure (in free nitrogen)  
Based upon both count and nitrogen determinations

A. Per cent oxygen in nitrogen.....	21	12.6	6.9	4.1	1.3
B. Final respiration rate, 0-10 hours.....	51	55	66	62	22
C. Initial respiration rate, 0-10 hours.....	24	22	22	20	15
$k$ (Based on count), 0-10 hours.....	0.363	0.630	1.17	2.70	11.5
$k'$ (Based on nitrogen content), 0-10 hours...	0.741	1.33	1.67	2.54	9.62

Initial count, 18 million per 2 cc.

Initial nitrogen, 1.2 mgm. per 2 cc. (as bacterial nitrogen).

3-day old *A. chroococcum* Strain SM 1, diluted 8 times.

$k'$  as in table 7.

maximum. The increase with time is sometimes negligible in free nitrogen, because, expressed in still another way, occasionally the second differential of relative growth is not appreciably greater than the second differential of relative respiration rate increase, with respect to time. In  $\text{NH}_3\text{-N}$ ,  $k$  invariably increases with

duration. If  $k$  were defined in terms of logarithmic functions of growth and growth respiration, it would, in general, increase in a linear fashion with time.

Recapitulating, we see that although the oxidation processes of the sort involved in temporal measurements of respiration rate bear qualitative functional relationships to the frequencies of cell division, there is no very fixed quantitative ratio under widely varying conditions of oxygen concentration, nitrogen supply, age of inoculum, humic acid concentration, and time.  $k$  varies but little in the case of most aerobic microorganisms, and probably does so in the case of *Azotobacter* because the maximum capacity to respire is so enormously high, three times its own dry weight of glucose per hour, as compared with values only one twenty-fifth as great in the case of baker's yeast (Warburg, 1927). That is, its possible range of variation of respiration rate between small and maximum values permits  $k$  to vary more than in the case of most organisms.

### III. THE CONCENTRATION OF FIXED NITROGEN REQUIRED TO INHIBIT FIXATION COMPLETELY

By growing *Azotobacter* in 21 per cent oxygen in, respectively, nitrogen and hydrogen, in different concentrations of rapidly available fixed nitrogen, it was found that the fractional increases in rates of respiration (final/initial rates) were the same in hydrogen and nitrogen for all concentrations of N above 0.5 mgm. per 100 cc., as shown in figure 5. Concentrations of N below this were not able to inhibit fixation completely, since growth was then greater in nitrogen than in hydrogen, i.e., nitrogen was no longer an inert gas. The values of the ratios of fractional increases in figure 5 are subject to a maximum inaccuracy of about  $\pm 8$  per cent, so that all values for concentrations of 0.5 mgm.  $\text{NH}_3$ -N per 100 cc. and above may be considered identical, within experimental error, whereas those at 0.2 and 0.1 mgm. are unquestionably higher. Other experiments described in Section IV confirm the inhibition concentration observed quantitatively. The abscissal values in figure 5 are plotted logarithmically in order to reduce spread. That the

inhibition value is independent of the concentration of bacteria in infinite dilution ranges is shown by the fact that cultures diluted three and fifteen fold give about the same ratios, over the fixed nitrogen range of concentration observed. Obviously, with very thick, heavy, old cultures, the inhibition value would be more difficult to determine, since the initial concentrations of

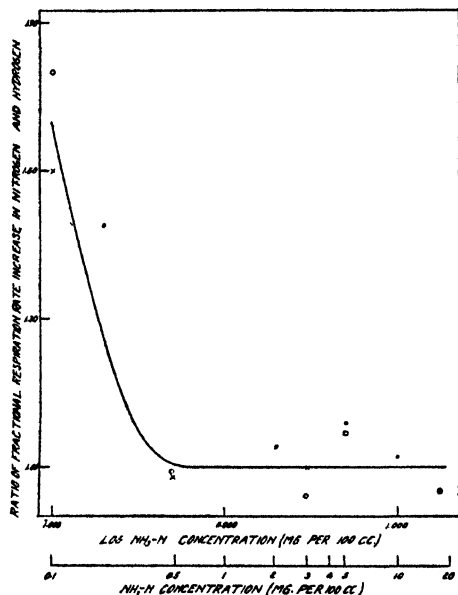


FIG. 5. FIXATION INHIBITION AS A FUNCTION OF FIXED NITROGEN CONCENTRATION

(•), the ratio (final/initial respiration rate) in  $N_2$  and  $H_2$ ,  $O_2$  pressure being kept constant at 21 per cent in each gas. Two-day culture of *A. vinelandii*, diluted 2 times. (□) is based on  $NO_3-N$  rather than  $NH_3-N$ . 7 hours duration.

(x), and (o), the ratio (final/initial respiration) in  $N_2$  and  $H_2$ ,  $O_2$  pressure being kept constant at 21 per cent in each gas. Three-day culture of *A. vinelandii*: (x), diluted three times; (o), diluted 15 times 6 hours duration.

fixed nitrogen added would soon decrease, and apparently higher values might be obtained.

The superiority of the present technique employed, with respect to the elucidation of fundamental characteristics of behavior, as compared with the usual extended, macro-experiments conducted without shaking to maintain equilibrium conditions, is clearly brought out. In the latter experiments, somewhat more

than the total amount of nitrogen the organisms can fix after a long period of time (one week or more) is found to be the completely inhibiting concentration, i.e., the integral rather than the differential effect is observed. The inhibiting integral concentrations so found are ten to twenty times the inhibiting differential concentrations observed by the writers. Thus, Kostyschew and coworkers (1926) found that slightly more than 15 mgm.  $\text{NH}_3\text{-N}$  per 100 cc. per gram of sugar (the amount of nitrogen the bacteria would otherwise have fixed) completely inhibited fixation. Kostyschew concluded that  $\text{NH}_3\text{-N}$  prevents fixation because (1)  $\text{NH}_3\text{-N}$  is the first product of fixation, and (2) the process is checked in accordance with the mass action law. This is hardly the case, however, as shown, indeed, by two aspects of Kostyschew's data, which the present writers themselves have confirmed. (1) The quantitative inhibition relations observed in  $\text{NH}_3\text{-N}$  are the same as in  $\text{NO}_3\text{-N}$ , a form of nitrogen which, according to Kostyschew, is not concerned in the mechanism, and therefore, presumably, not capable of mass action law effect against fixation. (2) The integral inhibiting concentration is too closely identical with the quantitative nitrogen requirements to mean other than that, as shown by figure 5, and numerous workers previously, the integral and differential limiting concentrations are determined solely by the nutritional needs of the bacteria as determined, in turn, by the capacity to divide and grow under the particular conditions of the environment. In this connection, rates as well as amounts must be considered. Thus, Kostyschew found that in the case of peptone nutrition even 130 mgm. of N per 100 cc. did not entirely prevent fixation; here, however, the nitrogen is much less rapidly available than in the case of  $\text{NH}_3\text{-N}$  or  $\text{NO}_3\text{-N}$ . Zoond (1926), incidentally, found a very much smaller amount of peptone, about one-fifteenth, to be completely inhibiting.

#### IV. GROWTH AS A FUNCTION OF FIXED NITROGEN CONCENTRATION

The rate of growth (ratio of final/initial respiration rate) as a function of rapidly available fixed nitrogen passes through a maximum at about 0.5 to 1 mgm. N per 100 cc., falling off sharply

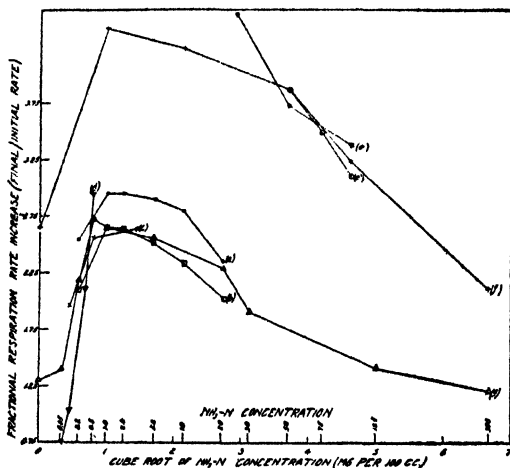


FIG. 6. GROWTH AS A FUNCTION OF FIXED NITROGEN

- a (•), 2 day culture, diluted 2 times, 7 hours duration, 21 per cent  $O_2$  in  $N_2$ . (x),  $NO_3-N$  instead of  $NH_4-N$ .  
 b (□), 2 day culture, diluted 2 times, 6 hours duration, 21 per cent  $O_2$  in  $H_2$ .  
 c (x), 3 day culture, diluted 2 times, 6 hours duration, 21 per cent  $O_2$  in  $H_2$ .  
 d (▽), 2 day culture, diluted 0 times, 24 hours duration, 21 per cent  $O_2$  in  $H_2$ .  
 e (\*), 1 day culture, diluted 2 times, 8 hours duration, 21 per cent  $O_2$  in  $N_2$ .  
 (e', (o),  $NO_3-N$  instead of  $NH_4-N$ .)  
 f (+), 2 day culture, diluted 2 times, 8 hours duration, 21 per cent  $O_2$  in  $N_2$ .  
 g (Δ), 3 day culture, diluted  $1\frac{1}{2}$  times, 7 hours duration, 21 per cent  $O_2$  in  $N_2$ .  
*A. vinelandii* used in all cultures.

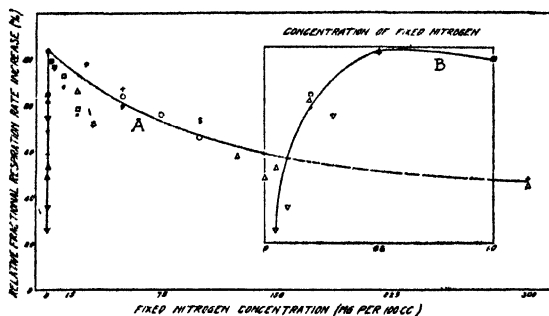


FIG. 7. GROWTH AS A FUNCTION OF FIXED NITROGEN

Curve A, replot of all curves of figure 6, on a relative percentage basis, as a direct rather than cube root function of concentration.

Curve B, same type of plot as in curve A, drawn to large scale to show abscissal portion of concentration between 0 and 1 mgm. N per 100 cc.

Points and experiments as in figure 6.

TABLE 9

*Total respiration as a function of fixed nitrogen*

Experiment a:										
Concentration of $\text{NH}_3\text{--N}$ (mgm. per 100 cc.).....	0	0.05	0.2	0.5	1	5	20	30	125	300
Total respiration (c.mm. of $\text{O}_2$ per 8 hours).....	397	438	466	570	550	548	512	418	404	318
Experiment b:										
(Concentration of $\text{NH}_3\text{--N}$ (mgm. per 100 cc.).....	0	1	25	50	100	300				
Total respiration (c.mm. of of $\text{O}_2$ per 8 hours).....	666	937	873	815	678	451				
Experiment c:										
(Concentration of $\text{NH}_3\text{--N}$ (mgm. per 100 cc.).....	0.2	1	2	10	20					
Total respiration (c.mm. of $\text{O}_2$ per 7 hours).....	964	1109	1071	1066	1036					
Experiment d:										
Concentration of $\text{NH}_3\text{--N}$ (mgm. per 100 cc.).....	0	0.05	0.1	0.3	0.5					
Total respiration (c.mm. of $\text{O}_2$ per 54 hours).....	31	66	81	140	232					
Experiment e:										
Concentration of $\text{NH}_3\text{--N}$ (mgm. per 100 cc.)... ..	0	0.05	0.1	0.3	0.5					
Total respiration (c.mm. of $\text{O}_2$ per 54 hours).....	0	61	73	132	190					
Experiment f:										
Concentration of $\text{NO}_3\text{--N}$ (mgm. per 100 cc.)... ..	0	0.5	2.5	5	10					
Total respiration (c.mm. of $\text{O}_2$ per 7 hours).....	465	700	690	700	712					
Experiment g:										
Concentration of $\text{NH}_3\text{--N}$ (mgm. per 100 cc.)... ..	0.1	0.5	3.0							
Total respiration (c.mm. of $\text{O}_2$ per 6 hours).....	397	500	508							

a. 3-day culture diluted  $1\frac{1}{2}$  times, 21 per cent  $\text{O}_2$  in  $\text{N}_2$ .b. 2-day culture diluted 2 times, 21 per cent  $\text{O}_2$  in  $\text{N}_2$ .c. 2-day culture diluted 2 times, 21 per cent  $\text{O}_2$  in  $\text{H}_2$ .d. 2-day culture diluted 0 times, 21 per cent  $\text{O}_2$  in  $\text{N}_2$ .e. 2-day culture diluted 0 times, 21 per cent  $\text{O}_2$  in  $\text{H}_2$ .f. 2-day culture diluted 2 times, 21 per cent  $\text{O}_2$  in  $\text{H}_2$ .g. 3-day culture diluted 3 times, 21 per cent  $\text{O}_2$  in  $\text{N}_2$ .All cultures *A. vinelandii*.



at lower concentrations, but less rapidly at higher concentrations, as shown in either figures 6 and 7, based upon respiration rate increases, or table 9, based upon total respiration. The abscissal values in figure 6 are plotted as cube root functions in order to reduce spread; the fall in rate at higher concentrations is therefore much slower, and at lower concentrations, much higher, relatively, than would appear at first glance (see curve A, fig. 7).

The rate of growth between 0.5 and 10 mgm. N per 100 cc. is, for practical purposes, identical, the decrease at the latter concentration being only a few per cent (see especially experiment *f*, table 9, and curves a, b, g, fig. 6). Experiment *f*, table 9, also shows that maximum growth is reached by at least 0.5 mgm.  $\text{NO}_3\text{-N}$  as well as by 0.5 mgm.  $\text{NH}_3\text{-N}$  per 100 cc. Curve *e'*, figure 5, shows that high concentrations of  $\text{NO}_3\text{-N}$  cause decreases in rates of growth just as do high concentrations of  $\text{NH}_3\text{-N}$ , as in curve *e*, so that the inhibition is owing to N as such, rather than to the nature of the chemical compound in which it appears. The pH was, of course, always constant in these experiments. It is interesting to note that growth in 200 to 300 mgm. N per 100 cc. is less than in free nitrogen; this observation has been confirmed by count measurements also.

The fact that maximum growth occurs at about 0.5 mgm. N per 100 cc. confirms the previous finding that the same, or at least no greater, concentration causes complete inhibition of nitrogen fixation, and, moreover, supports the view that the inhibiting effect of higher concentrations is not based upon mass law action, but simply upon the maximum metabolic capacity for using nitrogen, whether free or fixed. Growth is, indeed, considerably greater at 0.5 mgm. fixed nitrogen per 100 cc. (see *k* values in Section II) than in free nitrogen, so that obviously, in agreement with the view of Bonazzi (1924) and many others, nitrogen fixation is a means of nitrogen nutrition resorted to only in cases of relative nitrogen starvation.

[That fixed nitrogen (either as  $(\text{NH}_4)_2\text{HPO}_4$  or  $\text{KNO}_3$ ) at no very great concentrations should cause decreases in the rate of division and the rate of its own utilization, is by no means easily understood, especially since the effect is independent of the kind

of rapidly available fixed nitrogen, the nature of the charge of the ion bearing the nitrogen, obvious antagonism effects, or general osmotic effects. Burk (1930) has given two general type explanations, based upon chain reaction kinetics and contact catalysis, of the similar case of inhibition of oxygen consumption by high oxygen pressures, which might likewise apply here in principle, although hardly in detail.

TABLE 9a

*The nitrogen content of azotobacter supplied free nitrogen and different amounts of fixed nitrogen*

Experiment 1:						
NH <sub>3</sub> -N added (mgm. per 100 cc.).....	2	2	4	4	6	6
Dry matter obtained (mgm. per 100 cc.)....	45.3	48.3	54.2	56.0	69.3	77.3
Total nitrogen obtained (mgm. per 100 cc.)..	5.00	5.24	6.35	6.47	7.80	7.93
Per cent nitrogen in dry matter.....	11.0	10.8	11.7	11.6	11.3	10.3
Experiment 2:						
NH <sub>3</sub> -N added (mgm. per 100 cc.).....	2	3	4	4	5	
Dry matter obtained (mgm. per 100 cc.)....	44.4	52.8	60.7	59.6	70.4	
Total nitrogen obtained (mgm. per 100 cc.)..	3.55	4.31	5.22	5.22	6.08	
Per cent nitrogen in dry matter.....	7.9	8.2	8.6	8.8	8.6	

All experiments with *A. chroococcum* Strain SM 1, grown in 1-liter Roux flasks in air thermostat at 28°C. 75 cc. culture medium per flask, filling flask to depth of 3 mm. 1 drop of inoculum of young culture used. Initial concentration of sugar 1 per cent; sugar in no case entirely consumed. The added NH<sub>3</sub>-N was in all cases consumed before end of experiment. Nitrogen determined by Pregl micro-kjeldahl method, dry matter by centrifuging 20 cc. portions of culture, decanting supernatant fluid, drying precipitate *in vacuo* at room temperature and weighing to the fourth decimal place.

Experiment 1, culture grown for 10 days.

Experiment 2, culture grown for 6 days.

The view that nitrogen fixation is determined by the metabolic capacity for using nitrogen whether free or fixed is further supported by the data given in table 9a, based upon a different technique. It will be seen that in spite of the different amounts of nitrogen supplied (and the consequently different amounts of growth occurring), the nitrogen content of the dry matter is practically constant, i.e., the organisms have fixed nitrogen only so far as their normal needs require.

It has already been shown (Burk, 1930) that although humic

acid greatly stimulates the observed rate of nitrogen fixation, its action is not upon the process of fixation directly, but merely upon cell division or growth, the velocity of which to some extent determines the rate of fixation. The growth of organisms in fixed nitrogen is equally subject to stimulation by humic acid, as are also the other functions of initial respiration rate, final respiration rate, total respiration, growth, and efficiency of growth.

V. TOTAL RESPIRATION, GROWTH, AND EFFICIENCY OF GROWTH  
AS FUNCTIONS OF LIMITING CONCENTRATIONS OF FIXED  
NITROGEN

It has been found that with infinite dilutions of *Azotobacter* (0 to 200 million per cubic centimeter) the maximum rate of growth is reached at concentrations of 0.5 to 1 mgm.  $\text{NH}_3\text{-N}$  per 100 cc. Corresponding growth efficiency measurements were therefore determined from this approximate region of concentration down to zero concentration. A three-day old culture of *A. chroococcum* Strain SM 1, diluted 3 times with inorganic nutrient solution, made up to 0.5 per cent glucose, and containing 22 million bacteria per 2 cc., was grown in 6 different concentrations of  $\text{NH}_3\text{-N}$  in a gas containing 10 per cent  $\text{O}_2$  in  $\text{H}_2$ , for nine and twelve hours respectively (twelve simultaneous sub-experiments in all). Air as a source of oxygen was not used, in order to avoid the complicating effects of nitrogen fixation by organisms growing in markedly limiting concentrations of  $\text{NH}_3\text{-N}$ , and 10 per cent  $\text{O}_2$  was employed as a compromise between the normal value, 21 per cent, and 5 per cent, where growth (in  $\text{NH}_3\text{-N}$  as well as  $\text{N}_2\text{-N}$ ) is at a maximum with respect to oxygen concentration.

Table 10 gives the experimental results and table 11 the efficiency calculations. The results for 9 to 12 hours were obtained by subtracting those at 0 to 9 hours from those at 0 to 12 hours, making virtually three different experiments in all. The weighted average values have always been determined by giving double weight to 0 to 12 hours, where the experimental accuracy is highest. The observed total respiration, which is composed of respiration by both the new growth and the initial inoculum, is corrected for the latter by subtracting the total respiration

observed at 0.00 mgm.  $\text{NH}_3\text{-N}$  per 100 cc., the control in which no growth occurred. Efficiency calculations based upon respiration due to growth only (growth respiration) are the more fundamental and absolute numerically, although qualitatively the uncorrected respiration values lead to identical conclusions. The

TABLE 10  
*Growth and respiration as functions of limiting concentrations of fixed nitrogen  
(experimental data)*

Concentration of $\text{NH}_3\text{-N}$ (mgm. 100 cc.).....	0 00	0 02	0 10	0 30	0.50	5 0
Count increase (millions per 2 cc.) (initial = 22 millions per 2 cc.):						
0- 9 hours.....	0	2	6	38	54	66
0-12 hours.....	0	4	16	78	146	358
9-12 hours.....	0	2	10	40	92	292
Weighted average.....	0	3.0	12.0	58.4	109.6	268
Total respiration (c.mm. $\text{O}_2$ per 2 cc.):						
0- 9 hours.....	184 (193)	238 (240)	300 (287)	294 (296)	312 (306)	302 (307)
0-12 hours.....	261	325	414	466	493	585
9-12 hours.....	77	87	114	172	181	283
Weighted average.....	196	244	310	349	370	414
Growth respiration (respiration due to growth) (c.mm. $\text{O}_2$ per 2 cc.):						
0- 9 hours.....	0	54	116	110	128	118
0-12 hours.....	0	64	153	205	232	324
9-12 hours.....	0	10	37	95	104	206
Weighted average.....	0	48	115	154	174	243

method of correction used in the present experiments is exact, since, (1) no growth occurred in the control, and (2) the respiration rate per hour in the control remained constant during the whole experiment, indicating complete freedom from abnormal behavior. In evaluating the results, it must be observed that the experimental inaccuracy was about  $\pm 4$  per cent in the most

significant and instructive range (0.1 to 0.5 mg.  $\text{NH}_3\text{-N}$ ), but very much larger for 0.02 mgm.  $\text{NH}_3\text{-N}$ .

Table 10 shows the continuous increase of count, total respiration, and total respiration increase, with  $\text{NH}_3\text{-N}$  concentration and time. The figures in parenthesis for total respiration, 0 to 9 hours, are those of the simultaneously grown 0 to 12 hour cultures, and are given to indicate the degree of duplication obtained. The agreements at the different increasing concentrations of  $\text{NH}_3\text{-N}$  agree to within, respectively, 4.9, 0.8, 4.3, 0.6, 2, and 1.6, or an average of, 2.4 per cent.

TABLE 11  
*Efficiencies of growth as functions of limiting concentrations of fixed nitrogen  
(calculated data)*

Concentration of $\text{NH}_3\text{-N}$ (mgm. per 100 cc.)...	0 00	0.02	0.10	0.30	0 50	5.00
Count increase						
Total respiration:						
0- 9 hours.....	0	0 008	0 020	0.130	0.172	0.222
0-12 hours.....	0	0.012	0.038	0.168	0.296	0.612
9-12 hours.....	0	0.024	0.088	0.234	0.510	1.032
Weighted average.....	0	0.014	0.046	0.176	0.318	0.620
Count increase						
Growth respiration:						
0- 9 hours.....	0	0 036	0.052	0.346	0.422	0.560
0-12 hours.....	0	0 062	0.104	0.380	0.630	1.106
9-12 hours.....	0	0.200	0.272	0.422	0.886	1.418
Weighted average.....	0	0.090	0.132	0.382	0.642	1.048

Table 11 shows the continuous increase of efficiency (cell increase in millions per 2 cc./respiration in cubic millimeters per 2 cc.) with concentration of  $\text{NH}_3\text{-N}$  and time, for all concentrations. The magnitude of the effect is indeed striking, a 10 to 20 fold increase occurring between 0.02 and 5 mgm.  $\text{NH}_3\text{-N}$  in the case of the growth respiration efficiency, and a 25 to 50 fold increase occurring in the case of the total respiration efficiency.

The straight line function of efficiency of growth with respect to concentration of rapidly available fixed nitrogen, whether based upon total or growth respiration, is shown in figure 8,

curves II and I, respectively, using weighted average efficiencies for the three experiments in each case. Smoothed straight lines may be drawn for 0 to 9, 0 to 12, and 9 to 12 hour experiment efficiencies, as well as for their unweighted average, the least deviation of points from the line being obtained at 0 to 12 hours, where the experimental error is least.

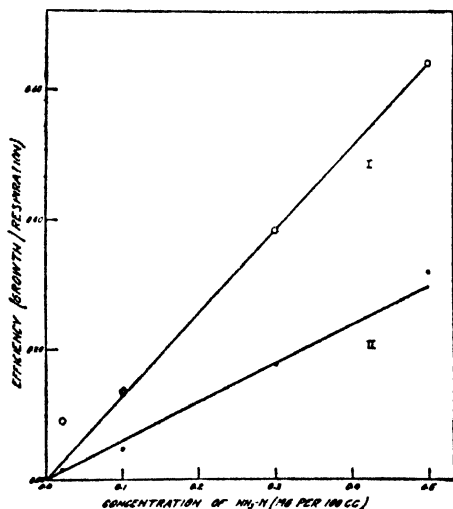


FIG. 8. EFFICIENCIES AS A FUNCTION OF LIMITING CONCENTRATIONS OF FIXED NITROGEN AND GROWTH

Curve I, weighted average efficiency, based upon respiration due to growth only, plotted against  $\text{NH}_3\text{-N}$  concentration.

Curve II, weighted average efficiency, based upon total respiration (i.e., including respiration of inoculum also), plotted against  $\text{NH}_3\text{-N}$  concentration.

Curve III, weighted average efficiency, based upon growth respiration, plotted against growth (count increase).

Curve I, figure 9, a replot of curve II, figure 8, to a larger scale, shows that maximum efficiency with respect to  $\text{NH}_3\text{-N}$  concentration is obtained at the same point on the abscissa where maximum growth is obtained, i.e., 0.5 to 1 mgm.  $\text{NH}_3\text{-N}$  per 100 cc. Curve II, figure 9, a plot of weighted average growth against  $\text{NH}_3\text{-N}$  concentration, shows that the relation is also a straight line up to nearly maximum growth. The quantitative ordinate differences between efficiencies (and likewise growths)

at concentrations of 0.5 and 5 mgm.  $\text{NH}_3\text{-N}$  are in some degree apparent, since the relative decrease in concentration from the initial has been greater in the former.

Figure 10 shows the extremely important fact that the efficiency of growth depends markedly upon the rate of growth. The relation is not quite linear, but is slightly concave downward.

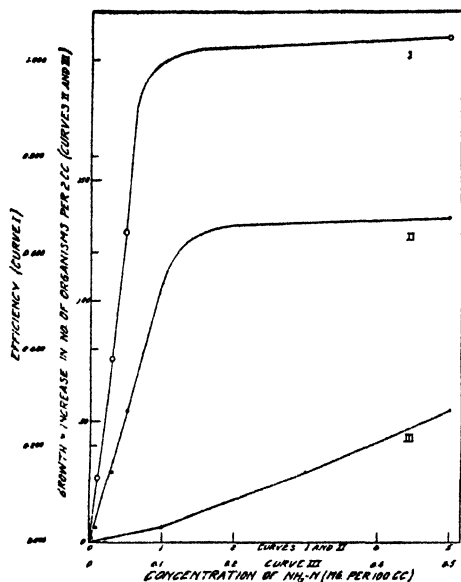


FIG. 9. EFFICIENCY AND GROWTH AS FUNCTIONS OF LIMITING CONCENTRATIONS OF FIXED NITROGEN

Curve I, replot of curve I, figure 1, on smaller scale.

Curve II, weighted average growth plotted against  $\text{NH}_3\text{-N}$  concentration.

Curve III, weighted average growth plotted against  $\text{NH}_3\text{-N}$  concentration, on larger scale.

This is the case, also, when growth is governed by limiting pressures of free nitrogen, even though growth when so governed is itself linear with respect to nitrogen pressure (Burk, 1930), just as growth in fixed nitrogen is linear with respect to limiting concentrations of fixed nitrogen.

Growth respiration, in distinction to growth and efficiency, does not vary with fixed nitrogen concentration directly, but as

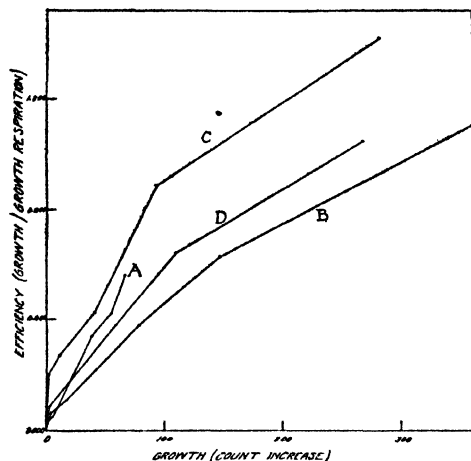


FIG. 10. EFFICIENCY OF GROWTH AS A FUNCTION OF GROWTH

Curves A, B, C, D, efficiencies based upon growth respirations at, respectively, 0-9 hours, 0-12 hours, 9-12 hours, and for weighted average values (see table 11).

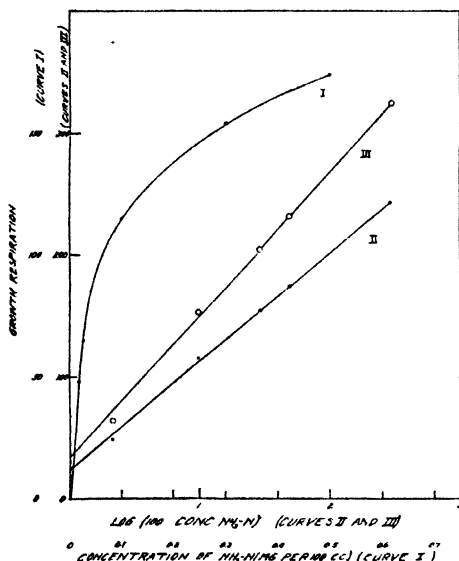


FIG. 11. GROWTH RESPIRATION AS A FUNCTION OF LIMITING CONCENTRATIONS OF FIXED NITROGEN

Curve I, weighted average growth respiration plotted directly against  $\text{NH}_3\text{-N}$  concentration.

Curve II, weighted average growth respiration plotted against logarithm of  $\text{NH}_3\text{-N}$  concentration.

Curve III, growth respiration, 0-12 hours, plotted against logarithm of  $\text{NH}_3\text{-N}$  concentration.



its logarithm. This is shown in figure 11, curves II and III, plots of, respectively, weighted average growth respiration and 0 to 12 hour growth respiration against logarithm (100  $\text{NH}_3 - \text{N}$  concentration). Curve I shows weighted average growth respiration plotted directly, rather than logarithmically, with respect to  $\text{NH}_3 - \text{N}$  concentration.

The physiological characteristics of growth behavior of *Azotobacter* maintained in limiting, non-inhibiting, concentrations of rapidly available fixed nitrogen may be summarized analytically as follows. If, for any given duration of time,  $x$  is the growth (in millions of bacteria per 2 cc.), and  $y$  the growth respiration (in cubic millimeters of oxygen per 2 cc.), when  $N$  is the concentration of  $\text{NH}_3 - \text{N}$  (in mgm. per 100 cc.) then

$$\begin{array}{lll} x = a N & (\text{Fig. 9}) & (1) \\ x/y = b N & (\text{Fig. 8}) & (2) \\ d(x/y)/dx > 0; d^2(x/y)/dx^2 < 0 & (\text{Fig. 10}) & (3) \\ y = c \ln N + d & (\text{Fig. 11}) & (4) \end{array}$$

where  $a$ ,  $b$ ,  $c$ , and  $d$  are constants, which depend, incidentally, chiefly upon the duration of the experiment, i.e., time. Equation (2) could not hold if equations (1) and (4) are valid, were it not for the fact that  $d(x/x^\circ)/dN$  is very much greater than  $d(y/y^\circ)/dN$  (where  $x^\circ$  and  $y^\circ$  are the count and respiration, respectively, when  $N = 0$ ), so that the latter is practically (i.e., experimentally) a constant with respect to the former. This may be seen from table 10 where between concentrations of 0.02 and 5 mgm.  $N$  the weighted average growth increases 89 fold (268/3) whereas the weighted average growth respiration increases only 5 fold (243/48), or a relative ratio of 18 fold (89/5). This, in itself, is a remarkable circumstance, and expresses, in still another way, after the manner of variation of both  $k$  and efficiency of growth with rate of growth, that, in *Azotobacter*, growth and growth respiration are by no means parallel functions.

If smoothed curves of efficiencies of growth are plotted against  $\text{NH}_3 - \text{N}$  concentration for each of the three experiments (rather than for their weighted averages in figure 8, curve I), the values of  $b$  in Equation (2) are found to increase somewhat with time, that is,  $d((x/y)/dN)/dt > 0$ , where  $t$  is time. So far as can be

determined,  $d^2((x/y)/dN)/dt^2$  is zero. Also, as is very obvious from inspection of table 11, for any given  $\text{NH}_3\text{-N}$  concentration the efficiency increases with time, that is,  $d(x/y)/dt > 0$ . As pointed out before, when considering  $k$ , in fixed nitrogen the second differential of fractional growth is always greater than the accompanying second differential of fractional growth respiration.

The equally marked dependence of efficiency of growth upon oxygen pressure, increasing ten to twenty fold between 0.21 and 0.001 atmosphere, whether the organisms are grown in either free or fixed nitrogen, is to be clearly distinguished from its dependence upon rate of growth at constant oxygen pressure. In the range of oxygen pressure where the efficiency of growth is increasing markedly, that is, below, 0.05 atmosphere, the growth rate is actually decreasing. In this range, the rate of growth respiration is decreasing much faster than the rate of growth, which of course, accounts for the efficiency itself increasing. One can perhaps picture the oxygen pressure effect on efficiency as concerned chiefly with the respiration enzyme, possibly in the region of the cell wall, the growth rate effect on efficiency, however, exerting its influence inside the cell at the locus where the mechanism of division takes place. The very great bearing of the relationship of the rate of growth to efficiency of growth upon the energetics of nitrogen fixation will be considered elsewhere.

#### SUMMARY

1. The equilibrium concentration of rapidly available fixed nitrogen in the culture medium required to inhibit nitrogen fixation by *Azotobacter* completely is 0.5 mgm. per 100 cc.
2. The rate of growth as a function of concentration of rapidly available fixed nitrogen passes through a maximum at a concentration of 0.5 to 1 mgm. per 100 cc., falling off sharply at lower concentrations, but much less rapidly at higher concentrations.
3. The efficiency of growth in fixed nitrogen depends markedly upon the rate of growth. Hence, although increases in rates of respiration with time may be used as a qualitative measure of

the amounts of growth occurring simultaneously, the constant of proportionality,  $k$ , between growth and growth respiration rate increase increases with the rate of growth, which in turn may depend upon pressure of nitrogen gas, concentration of fixed nitrogen, presence of humic acid, oxygen concentration, age of inoculum, and time.

4. The percentage composition of nitrogen in the dry matter of the cells varies little whether the nitrogen supply is chiefly fixed or free.

5. The view of previous workers is supported and enlarged upon, that fixation is a function resorted to only in the absence of sufficiently available fixed nitrogen.

6. The various physiological functions of respiration, growth, efficiency, etc., of *Azotobacter* maintained in free and fixed nitrogen have been compared qualitatively. The behavior is quite similar in the two cases, and no conclusions may be drawn, therefore, concerning the chemical mechanism of nitrogen fixation.

The writers are much indebted to Dr. F. E. Allison, Dr. W. E. Deming, Dr. Carl Iddings, and Dr. F. M. Schertz for valuable criticism.

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# A NEW VESSEL FOR THE EFFICIENT AERATION OF BACTERIAL CULTURES IN LIQUID MEDIA

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## INTRODUCTION

In a study of the fundamental physiology of microörganisms it is essential that all the individuals of the culture population be subjected, as nearly as possible, to uniform environmental conditions. Not only must the organic and mineral constituents of the medium be made available to all cells, but the gaseous environment and temperature must be controlled; and the products of metabolism, if not removed, must be uniformly distributed. These conditions are usually far from being realized in the ordinary bacterial culture. In the case of a bacterial colony on a solid medium, for example, the lower layers of organisms lie in intimate contact with the food material. As development of the colony proceeds, however, the oxygen supply becomes more restricted and the products of metabolism, elaborated by these organisms and those massed above them, pollute and poison the food supply. Those on the surface, on the other hand, have ready access to the oxygen supply, but they must depend for their food upon either the movement upward of nutrients through the masses of organisms beneath, or, wholly or in part, on the products of their metabolism. Only on the very edges of the colony does anything approaching a satisfactory condition exist; and even here the medium is often heavily infiltrated with bacterial wastes or other products of colony activity. The situation is comparable to that

<sup>1</sup> The writers are indebted to Mr. Leonardo Testa of Washington, D. C., whose technical skill in constructing laboratory equipment made possible the development of this vessel.

of an hypothetical city, a thousand times more congested than any now known, with food and water supplies polluted, ventilation unprovided for, and no sewage disposal facilities—a city of individuals piled one upon another and cut off from practically everything conducive to what is commonly thought of as a normal existence.

Even when liquid media are used, conditions often are hardly more satisfactory. This is particularly true where pellicle-forming or strictly aerobic and anaerobic types of organisms are being grown. To be sure, the products of metabolism may be removed somewhat more rapidly by diffusion and convection, but, in spite of this, it is often true that the upper portions of the medium have an entirely different reaction from those deeper in the culture vessel; and discoloration of these upper regions by soluble pigments or other substances are often observed, showing that here the products of metabolism are more concentrated.

A student of human physiology could hardly use with satisfaction the population of the hypothetical city just mentioned as the subject of his experiments on the fundamental needs of human beings, even if it were possible to do so; and it would seem equally unsafe to base conclusions, as to the fundamental physiology of any bacterial species, on the results obtained with cultures grown under conditions so thoroughly uncontrolled. Indeed, the wide variation in results of experiments, and the fact that they are sometimes irreproducible, are in large part to be explained on the grounds of inadequate cultural control.

Bacteriologists, and others, are vitally interested, of course, in the products of mass activities of microorganisms. In Nature, ideal laboratory conditions probably rarely, if ever, exist. Bacteriological workers, in their study of species, will continue to make use of the time-honored and useful, though inexact, cultural methods now employed; but it is believed that more rapid and satisfactory progress will be made in the field of bacteriological research as new means are found to bring under more complete control the factors influencing bacterial metabolism.

## THE NEW CULTURE VESSEL

In the studies of the writers on sporulation among bacteria and the thermal resistance of bacterial spores the need for more exact

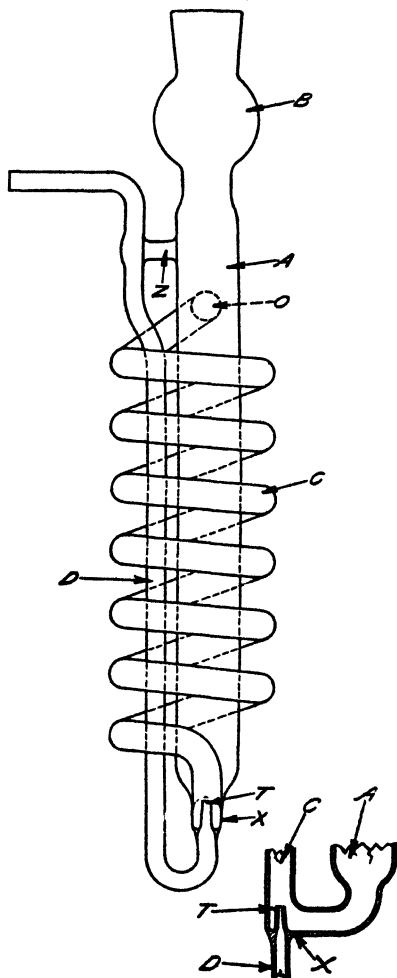


FIG. 1. THE CULTURE VESSEL

methods for culturing the organisms has been found imperative. As a result of repeated endeavors to meet this need, a new culture vessel has been developed which has shown so much promise of

fulfilling the requirements that a description and illustration of it are presented here. It provides a convenient receptacle for the culture; it is so constructed that the degree of aeration may be controlled at will; the temperature throughout the culture is made uniform; and the entire contents of the vessel are kept in gentle but constant circulation, thus assuring uniform environmental conditions for all organisms within the culture. The illustration of this new culture vessel is presented in figure 1.

The device as illustrated consists of a central vertical tube (A) about  $\frac{3}{4}$  inch in diameter and 10 inches long, enlarged near the top into a bulb (B) having a neck of sufficient length to receive the usual cotton plug, and communicating at the base with a spiral tube (C) having an internal diameter of about  $\frac{1}{4}$  inch, which loosely circles the central vessel in a series of coils and opens into it at the point (O) a short distance below the bulb. At the base of the spiral tube, designated as point (X) in the illustration, an air-inlet tube (D) of about  $\frac{3}{8}$  inch internal diameter is inserted and fused which has a tip (T) about  $\frac{1}{4}$  inch long projecting upward into the spiral (see also offset detail drawing). The body of this tube parallels the walls of the central chamber within the circle of the spiral coils and is supported by the solid bar (Z) which is fused at one end with the wall of the air-inlet tube and at the other end with the wall of the central vessel. The upper end of the air-inlet tube is bent to a right angle to facilitate attachment with the compressed air, or other gas supply, and the arm is of sufficient length to receive a small cotton plug.

The complete vessel as shown here has a working capacity of about 75 ml., though the capacity may be varied, of course, by the use of larger or smaller tubing in its construction.

Pyrex glass has been employed in the manufacture of the vessels used by the writers. This avoids the possible alteration of the hydrogen-ion concentration of the medium through absorption of basic substances from the glass, an important consideration where careful work must be done, as has been pointed out by Esty and Cathcart (1921), and reduces the breakage which is often so disastrous to an experiment. The apparatus described is sturdy and will stand ordinary laboratory handling with a minimum of breakage.

## OPERATION

Since sterilization of the medium within a culture tube often results in partial loss of water, and consequent modification of the concentration of the nutrient constituents of the medium, it has been the practice of the writers to sterilize the medium and culture vessel separately and then to distribute the medium to the culture tubes and vessels, using aseptic precautions. The sterilization of this vessel is carried out in the usual manner, either dry in the hot air oven or moist in the autoclave. Because of saving in time and less danger of breakage the writers have preferred the latter method. Before sterilization, both openings of the vessel are plugged with cotton. When sterilized, the medium is introduced, with aseptic precautions, until the level of the liquid reaches a little above the outlet (*O*). Inoculation is now made and the culture vessel is ready to be set up within the incubator. The vessel may be suspended by wire from a bar, held by a clamp or placed in a suitable support. It is desirable that the tip from which the bubbles of air or gas emerge be visible for the careful regulation of the rate of aeration. The cotton plug of the air-inlet tube is now flamed flush with the end of the tube and left in place, the rubber tube connection with the air or gas supply being slipped on over it. Both cotton plugs must be dry; of course, to allow of the movement of the air through them. All that remains to do is to regulate the gas flow, which must be controlled by needle valve or other delicately adjusting device. Compressed air, artificial gas mixtures or inert gas may be used as desired. If found desirable to pass the air or gas through a scrubber a second vessel like the one described will be found most effective.

A thorough aeration of the culture results from the continuous liberation of bubbles of air or gas into the base of the spiral and their passage upward to the top of the tube. Not only is the culture brought into intimate contact with bubbles but the entire culture is kept in gentle but constant circulation, due to the fact that the bubbles in rising force the liquid upward with them. The rate at which this aeration and circulation takes place depends, of course, upon the rate of bubbling.



Positive circulation takes place effectively in the vessel as described when bubbles are introduced at the rate of one bubble per second. At this rate 10 bubbles totaling 1 ml. in volume are constantly in contact with the medium. At this rate of bubbling a complete circulation of the entire culture has been found to take place once every fifty-five seconds. A faster rate of bubbling brings more bubbles into contact with the medium and shortens

TABLE 1

*Showing effect of the rate of bubbling on the time required to complete circulation of medium on the number of bubbles constantly in contact with the medium and their total volume*

BUBBLES PER MINUTE	SECONDS TO COMPLETE CIRCULATION	NUMBER OF BUBBLES IN CONTACT WITH MEDIUM	TOTAL VOLUME OF GAS IN CONTACT WITH MEDIUM
			ml.
30	97	6	0.6
40	78	8	0.8
50	65	9	0.9
60	55	10	1.0
70	47	11	1.1
80	41	12	1.2
90	37	13	1.3
100	35	14	1.4
120	31	17	1.7
140	28	19	1.9
160	25	21	2.1
180	22	22	2.3
200	20	24	2.5
240	17	27	3.5
280	13	29	5.0

the period of complete circulation accordingly. The rate and completeness of circulation is dependent to some extent on the size of the bubbles as well as on the rate at which they emerge from the tip. Under working conditions as here set forth, the average size of bubbles formed at the operation rate of sixty bubbles per minute was found to be one-tenth of 1 ml. and the number of bubbles actually in contact with the medium for this rate was ten. In other words, 1 ml. of air in the form of ten bubbles was constantly in contact with the medium. At rates of operation

above about 170 bubbles per minute the bubbles tended to coalesce progressively as the rate of bubbling was increased, thus reducing, relatively, the aerating surface exposed. The movement of the medium through the vessel was correspondingly increased, however, so that abundant aeration was secured.

Table 1 is based on numerous observations showing the effect of different rates of bubbling on the time required for complete circulation of the medium and on the number of bubbles and their total volume in the medium at one time.

#### DISCUSSION

The use of the spiral in gas scrubber and gas absorption vessels is not new, the principle being applied in the Milligan gas absorption apparatus which made its appearance several years ago. It was also made use of in a somewhat different way by Bishop (1923) in his study of the body fluid of the honey bee larva. These vessels, however, are made up of several units which, in the experience of the writers, makes them particularly liable to breakage and difficult to sterilize satisfactorily.

The culture vessel here described is a single unit and is easily cleaned, sterilized and handled. It is so constructed that samples of the medium may be readily removed for chemical or other examination, and by substituting a suitable stopper and tube in the place of the cotton plug gases may also be collected for analysis. The vessel is particularly well adapted to the cultivation of pellicle formers as the organisms are kept in constant motion and pellicles do not form. In operation, it provides for thorough aeration of all organisms in the culture, assures the even distribution of all products of metabolism, and in the laboratories of the writers has solved a number of difficulties hitherto encountered in controlling the growth and activities of bacteria under study.

#### CONCLUSION

A new culture vessel is described and illustrated which in operation provides for the thorough aeration of cultures, assures the uniform distribution of the products of metabolism, and facilitates the study of bacterial activities.

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# THE INHIBITION OF PHYTOMONAS MALVACEARA IN CULTURE MEDIA CONTAINING SUGARS

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## INTRODUCTION

In some experiments performed recently to determine the various sources of carbon and nitrogen suitable for the growth of *Phytomonas malvaceara*, it was discovered that certain sugars cause complete inhibition of growth under some conditions but form satisfactory sources of carbon under other conditions. Inhibition appears to depend on the composition of the culture medium, the amount of sugar present and the method of sterilization.

The mineral base to which the carbon and nitrogen compounds were added was prepared according to the recent formula of Frazier and Rupp (1928). Two solutions were employed depending on whether ammonia or some other source of nitrogen was to be supplied. The nitrogen free solution contained dibasic potassium phosphate 0.31 per cent, monobasic potassium phosphate 0.08 per cent and 0.02 per cent each of potassium chloride and magnesium sulphate. The salts were dissolved in double distilled water. In the medium used for testing utilization of ammonia, 0.25 per cent of sodium ammonium phosphate was substituted for the potassium phosphates.

Three carbon compounds were used to test for ability to utilize ammonia. These were sucrose, glucose and glycerol. One per cent of each was added separately to the sodium ammonium phosphate solution. The resulting media were then distributed, in about 5 cc. quantities, in pyrex test tubes and sterilized at 122°C. for fifteen minutes.

Fourteen strains of *Phytomonas malvaceara* freshly isolated from infected leaves of cotton were used for inoculation. Each strain was tested for purity, conformation to type description, and pathogenicity before beginning the experiments. All inoculations were made with a single loopful of an aqueous suspension prepared from forty-eight-hour agar slant cultures.

In the medium containing glucose, no growth had occurred at the end of a prolonged incubation period of thirty days while in the media containing sucrose or glycerol growth was prompt and vigorous. It is obvious that these results might be due to a variety of causes. If the organism is not able to use ammonia as a source of nitrogen it might obtain sufficient nitrogenous material from impurities in the sucrose and glycerol but not from the glucose. This appeared to be the most probable explanation but one which was found untenable. Neither sucrose nor glycerol supported growth when added to the nitrogen free mineral solution. Thus, it would appear that the organism is able to use ammonia as a source of nitrogen and either sucrose or glycerol but not glucose as a source of carbon.

It is well known that organisms are variable both as to nitrogen and carbon requirements. Honing (1913) reported growth of a greater number of strains of *B. solanacearum* with either sucrose or glycerol than with glucose. The results of other studies appear to show, however, that glucose is generally more satisfactory as a source of carbon than either sucrose or glycerol and it has been extensively used as the carbon compound for testing utilization of various sources of nitrogen.

That failure of growth in the above experiment was not caused by lack of ability to use glucose was determined by filtering a solution of glucose and then adding it to the sterilized mineral base. In the medium prepared by this method, all of the strains grew promptly and with great vigor. Thus, it is shown that inhibitory substances produced by sterilization at high temperatures rather than inability to assimilate glucose is responsible for the failure of growth.

Since solutions containing sugars undergo changes in reaction when sterilized by heat it seemed possible that the high tem-

perature of sterilization might have raised the hydrogen ion concentration sufficiently to cause inhibition. That other chemical changes also occur was denoted by marked changes in color in the medium containing glucose but not in media containing sucrose or glycerol.

Although many workers have reported on the changes of reaction which occur in various solutions when subjected to heat, it seemed desirable to determine precisely how much change occurs in the media employed here. The two mineral solutions containing 1.0 per cent each of glucose were placed in specially cleaned pyrex flasks and heated at different temperatures for

TABLE 1

Showing hydrogen ion concentration of mineral solutions A and B containing 1.0 per cent glucose

TEMPERATURE		pH OF SOLUTIONS												
		Time in minutes												
		0	5	10	15	20	25	30	40	50	60	70	80	90
°C.														
100	B	7.66	7.56	7.43	7.38	7.31	7.26	7.15	7.08	6.97	6.89	6.72	6.68	6.64
115.5	A	7.15	7.11	7.08	7.03	6.96	6.89	6.73						
	B	7.66	7.42	7.38	7.31	7.28	7.23	7.21						
122	A	7.15	7.06	7.03	6.96	6.86	6.84	6.78						
	B	7.66	7.38	7.35	7.14	6.97	6.94	6.88						

various periods of time. The reactions were determined electrometrically, both before and after heating. The results are shown in table 1. The nitrogen free solution is designated in the table as solution A, the sodium ammonium phosphate solution as solution B. It is seen that the hydrogen ion concentration increases in all cases but that the final reaction even after a period of thirty minutes at 122°C. is only slightly acid, about pH 6.8. *Phytomonas malvaceara* grows very well in peptone beef extract broth adjusted to a reaction of pH 5.4 with hydrochloric acid. There seems then no reason to believe that failure of growth in the synthetic medium containing glucose is due to the

reaction of the sterilized medium. It appears rather that sterilization of glucose at high temperature, in the mineral solution, causes some other chemical change which renders it unsuitable for utilization or detrimental to the growth of the organism.

Before continuing experimental investigation of this problem a search was made to determine whether such a phenomenon had been reported previously. Two instances only which seem to be comparable have come to my attention. Fisher and Bunte (1928) studied the effect of overheated media containing lactose. Milk was heated for a period of forty to sixty minutes at a temperature of 125° to 127°C. and then mixed with an equal volume of sterile 4.0 per cent agar dissolved in water. On this medium, *Salmonella schotmülleri* failed to grow while *Salmonella enteritidis* grew normally. The same result was obtained in a synthetic medium containing lactose 2.0 per cent, sodium citrate 0.2 per cent, ammonium sulphate 0.01 per cent and sodium phosphate 0.05 per cent.

Uyeda (1905) reported that *B. nicotiana* grew in a synthetic medium containing asparagin as the sole source of nitrogen and carbon but failed to grow in the same medium when glucose was added. Erwin F. Smith (1914) comments on this finding of Uyeda as follows: "That the latter which with asparagin made a weak growth, made none whatever when 1.0 per cent of glucose was added, should have indicated to Uyeda that bacteria are variable like other things, because, in the first place 1.0 per cent glucose is not a poison. . . . " Smith's comment fails to shed any light on the nature of the phenomenon for it is obviously not a case of variability in the utilization of a carbon compound but inhibition caused by it in a culture medium otherwise suitable for growth. In other words 1.0 per cent of glucose, whether it is a poison or not, suppressed growth completely. That *Phytomonas malvaceara* is similarly inhibited by 1.0 per cent of glucose and other sugars with various sources of nitrogen and in culture media which, without the sugars, support abundant growth will be shown in subsequent sections.

## EFFECT OF DIFFERENT METHODS OF STERILIZATION

Since glucose sterilized by filtration proved to be suitable for the growth of *Phytomonas malvaceara*, while no growth occurred in the medium sterilized at 122°C., additional experiments were performed to test the effect of other methods of sterilization. The sodium ammonium phosphate mineral solution plus 1.0 per cent of glucose was placed in pyrex tubes and sterilized either by heating in an Arnold sterilizer for thirty-minute periods on three successive days or by heating in an autoclave at 10 pounds pressure for a period of twenty minutes. There were no signs of inhibition in media sterilized by either of these methods. Thus, it appears that the temperature reached is of more importance than the total period of heating. It was also observed that the solutions sterilized at lower temperatures suffered much less marked changes in color. The medium was likewise suitable for growth when glucose was sterilized at 122°C. in distilled water or the mineral solution minus phosphate and then added to the sterilized mineral solution. No growth occurred when glucose and phosphate were sterilized together for fifteen minutes at 122°C. and then added to the solution containing potassium chloride and magnesium phosphate. Thus it appears that glucose and ammonium phosphate react together at high temperatures to form compounds which are inhibitory.

## EFFECT OF THE SOURCE OF NITROGEN

In the light of Uyeda's results with *B. nicotiana* it seemed desirable to test other sources of nitrogen to determine whether or not inhibition occurs in media which, without glucose, are suitable for growth. The list of compounds tested included asparagin, glutamic acid, alanine, glycine, beef extract 0.3 per cent each, tyrosine 0.1 per cent, and peptone 0.1 to 1.0 per cent. These were dissolved in the nitrogen free phosphate mineral solution which contained 1.0 per cent glucose and were then sterilized at 122°C. for fifteen minutes. Similar media without additional carbon and media containing sucrose or glycerol were prepared.



Four of the compounds, peptone, beef extract, glutamic acid and alanine supported growth in the absence of additional carbon. All of the sources of nitrogen proved suitable in the presence of either sucrose or glycerol. In the media containing glucose, no growth occurred except with peptone and, here, only when the amount was greater than 0.2 per cent. The time of observation extended over a period of thirty days. In peptone glucose media containing either 0.1 or 0.2 per cent peptone and 1.0 per cent glucose but without phosphate, growth occurred promptly. Faint turbidity was evident within twenty-four hours, the growth becoming abundant within forty-eight to seventy-two hours.

These results show that in the presence of glucose and phosphate sterilized together at 122°C. for fifteen minutes, growth is completely inhibited in culture media which without glucose are suitable for growth. Similar results were obtained by adding 1.0 per cent each of glucose and sucrose to the sodium ammonium phosphate solution. With sucrose alone, growth was abundant but when glucose also was present no growth occurred.

#### EFFECT OF THE AMOUNT OF GLUCOSE AND PHOSPHATE

Since inhibition appears to be due to products formed by the reaction of phosphate with glucose the degree of inhibition should be proportional to the amount of these compounds present in the medium. The effect of the amount of glucose was tested in a solution containing peptone 0.1 per cent, dibasic potassium phosphate 0.5 per cent and glucose 0.1 to 1.0 per cent at intervals of one-tenth. Through the range from 0.1 to 0.4 per cent the organism grows as well as in the control tubes without phosphate. Above 0.6 per cent no growth ever occurred. It is evident, therefore, that inhibition is proportional to the amount of glucose present.

In a similar manner the effect of different amounts of phosphate was determined. In this case the basic medium contained peptone 0.1 per cent, glucose 1.0 per cent and dibasic potassium phosphate 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.40, and 0.50. No inhibition occurred when phosphate was present in amounts of less than 0.2 per cent. Above this amount no growth occurred.

**EFFECT OF HYDROGEN ION CONCENTRATION DURING STERILIZATION**

It has been shown that failure of growth is not due to the hydrogen ion concentration reached by the media during sterilization. Throughout the experiments it has proved impossible to distinguish between inhibitory and non-inhibitory media by differences in hydrogen ion concentration. It remains to be determined whether or not inhibitory substances are formed in acid media, sterilized and then adjusted to a point within the required range of the organism.

The effect of hydrogen ion concentration during sterilization has been determined. The medium contained peptone 0.1 per cent, dibasic potassium phosphate 0.5 per cent, and glucose 1.0 per cent. The reaction was adjusted to about pH 5.4 before sterilization at 122°C. for fifteen minutes. After sterilization the reaction was readjusted to pH 7.2. No change of color occurred during the heating period and all strains of the organism grew promptly and vigorously. In a lot of the same medium sterilized without adjustment to acid reaction no growth occurred.

In a medium containing 0.1 per cent phosphate, 0.1 peptone and 1.0 per cent glucose sterilized for fifteen minutes at 122°C. growth occurred promptly. In a lot of the same medium adjusted to about pH 8.8 before sterilization the reaction remained alkaline throughout but inhibitory substances were not formed. Similar results were obtained when alkalinity was maintained by calcium carbonate. Thus, it appears that inhibitory substances are not formed in alkaline solutions in the absence of phosphate. All of the evidence indicates that the inhibitory substance is produced by specific reactions between glucose nitrogen compound, and phosphate in alkaline media.

**THE EFFECT OF CAMELIZATION**

Since, in all alkaline solutions containing glucose, chemical changes were denoted by the production of a red-brown color, it seemed desirable to determine definitely whether caramelization of glucose alone by heat results in the formation of inhibitory substances. In this experiment 5.0 grams of glucose were

heated in an evaporating dish until the solution became very strongly caramelized. The resulting dark red-brown syrup was then diluted with 500 cc. of distilled water to which was added 0.1 per cent peptone and 0.1 dibasic potassium phosphate. The resulting medium brought to a reaction of pH 7.2 was then sterilized at 122°C. for fifteen minutes. All strains of *Phytomonas malvaceara* grew as well as in the control medium containing uncaramelized glucose.

#### EFFECT OF OTHER SUGARS

Four additional sugars, maltose, lactose, galactose and levulose have been tested. The medium contained peptone 0.1 per cent and dibasic potassium phosphate 0.5 per cent. The sugars were added in concentrations of from 0.1 to 1.0 per cent. The resulting media were then sterilized at 122°C. for fifteen minutes. None of the media supported growth in concentrations greater than 0.6 per cent of the sugar while levulose proved to be inhibitory at a concentration of 0.4 per cent. Levulose was found to be inhibitory when sterilized at 10 pounds pressure for twenty minutes.

#### INHIBITION OF OTHER SPECIES

Since *Phytomonas malvaceara* is so markedly inhibited by the products formed by sterilization of sugars in media containing phosphates, other species were tested for comparison. Six species capable of utilizing ammonia were tested in the sodium ammonium phosphate solution. *Serratia marcescens*, *Salmonella enteritidis*, *E. coli*, *Aerobacter aerogenes*, *Ps. fluorescens* and *Ps. aeruginosa* were not inhibited. In a medium containing peptone 0.1 per cent, beef extract 0.3 per cent, dipotassium phosphate 2.0 per cent, and glucose 5.0 per cent sterilized for thirty minutes at 122°C. some additional species were inhibited while others grew as promptly and vigorously as in the control medium which contained no glucose. The inhibited species included *Staph. albus*, *Staph. aureus*, *Sarcina lutea*, *B. mycoides* and *B. anthracis*. The non-inhibited species included *E. coli*, *B. subtilis*, *Aerobacter aerogenes*, *Serratia marcescens*, and *Ps. fluorescens*.

## THE RÔLE OF PHOSPHATE IN TRANSFORMATIONS OF HEXOSE SUGARS

The transformations which occur in hexose sugars due to the influence of caustic alkalis, lead hydroxide, calcium hydroxide, or sodium carbonate have been studied by Lobry de Bruyn and Van Ekenstein (1895) and by Nef (1914). These observers found that when either d-glucose, d-mannose or d-fructose was treated with these solutions a mixture containing all of these sugars resulted and in addition a 3-ketohexose was formed which they designated as d-glucose.

More recently Spoehr and Wilbur (1926) have shown that similar transformations occur when d-glucose or d-fructose is treated with dibasic or neutral sodium phosphate. They question the nature of the so-called d-glucose and offer the suggestion that it is probably a mixture of the nature of formose, acrose, and the condensation products of glyceric aldehyde and dihydroxyacetone. They regard it as a significant fact that "solutions of both d-glucose and d-fructose in the presence of disodium phosphate, in time become colored through the formation of tar." They found also that tar formation does not occur if there is present in the mixture an oxidizing or reducing agent. When the glucose-disodium phosphate mixture was reduced with aluminium amalgam, no tar resulted but acetone was formed as a product of reduction. This was offered as evidence of the splitting of the hexose into a molecule containing three carbon atoms, presumably glyceric aldehyde, which is converted into dihydroxyacetone or acetal. Either of these two compounds yields acetone on reduction. In the absence of a reducing agent or an oxidizing agent the splitting products polymerize, resulting in tar formation or condense to form an optically inactive mixture. It would appear then that the chief action of phosphate is due to the dissociating influence of the salt. This also explains the catalytic effect of disodium phosphate on the oxidation of hexoses.

To explain the transformation of glucose to methyl glyoxaline due to the action of strongly dissociated zinc hydroxide ammonia, Windaus and Koop (1905) assumed that glyceric aldehyde is first formed. Windaus (1907) found also that the reaction is

not confined to glucose but that the same methyl glyoxaline is yielded by mannose, fructose, sorbose, arabinose, xylose, rhamnose, and lactose.

The reactions which are known to occur at high temperatures in solutions containing phosphate, sugars, and nitrogen bearing compounds appear to afford an explanation for all of the phenomena observed in the present investigation. The phosphate acts as a buffer to maintain alkalinity during the heating period and influences dissociation of the sugar. The resulting aldehyde reacts with the nitrogen compound to form a new substance which is suitable as a source of nitrogen for some species but not for others. The original source of nitrogen is completely exhausted only when the amount is relatively small and in the presence of sufficient sugar and phosphate. The exact proportions of nitrogen bearing compound, sugar and phosphate vary with the temperature and period of sterilization.

#### SUMMARY AND CONCLUSIONS

1. *Phytomonas malvaceara* fails to grow in culture media containing glucose, maltose, lactose, galactose or levulose and various nitrogenous compounds when sterilized at 122°C. for fifteen minutes. This failure of growth is not due to lack of ability to assimilate the various sugars but to chemical changes which are caused by the high temperature of sterilization.

2. Inhibition depends on the amount of peptone, sugar, and phosphate present; the reaction of the solution during the heating period; and the temperature and length of the period of sterilization. It is not due to changes in hydrogen ion concentration during sterilization.

3. Inhibition occurs in culture media which, in the absence of the sugars, are suitable for growth.

4. The evidence seems to warrant the conclusion that inhibition is due to conversion of the nitrogen compound into a form which is not suitable for assimilation by some species of bacteria.

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